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A Complement-fixing Antibody in Sera of Rabbits Bearing Brown-Pearce Carcinoma.

F. S. Cheever. (Introduced by H. Zinsser.)

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Kidd[1] and Dmochowski[2] independently have reported the demonstration of a serologically active substance associated with extracts of the Brown-Pearce carcinoma.[3] Recently Kidd[4] has demonstrated the immunological specificity of the material and has outlined its more important physical properties. The analogy to the serologically active substance extracted from virus-induced rabbit papillomas is striking.[5] The Brown-Pearce carcinoma, however, resembles most mammalian tumors in that the immediate cause of the proliferative process is unknown; in particular no virus agent has been demonstrated. Thus Kidd's observations seemed sufficiently important to justify the repetition of his original serological experiments.

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[1] Kidd, J. G., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 292.

[2] Dmochowski, L., *Compt. rendue de la Soc. de Biol.*, 1938, **129**, 349.

Materials and Methods. The tumor was obtained through the kindness of Dr. J. B. Murphy in March, 1939. It was transplanted through a series of normal hybrid adult rabbits obtained from several dealers. The intratesticular route of inoculation was used. The material was obtained from vigorously growing testicular tumors or from metastases and was prepared essentially in the manner originally described by Brown and Pearce[3] with minor modifications. Recognizable takes developed in 78% of the cases. A few animals were inoculated simultaneously by several routes—intraperitoneal, intratesticular and intramuscular—in order to insure large masses of tumor developing at the same time. Another small group was immunized by Besredka's method of intracutaneous inoculation.[6] The animals were bled and sacrificed at some time between the 9th and 182nd days after inoculation. The tumor material was removed under aseptic precautions, freed of grossly necrotic elements and stored in Petri dishes at –20°C or under 50% glycerol-Locke's solution at 4°C according to the method outlined by Kidd.[4] Pathological sections were made of any grossly atypical tumor material before it was accepted as being a satisfactory antigen. Kidd's[4] directions were followed for the final preparation. The tissue was ground in a mortar with the gradual addition of isotonic saline to make a 1:20 suspension, permitted to stand overnight in the refrigerator and then spun twice at 3800 R.P.M. for 5 and 20 minutes respectively. The resulting slightly opalescent suspensions were inactivated for 30 minutes at 56°C immediately prior to use, as were the sera, which were obtained sterilely and stored without preservatives in the refrigerator. The actual test was set up using 0.2 cc of 1:4 dilution of serum, 0.2 cc of complement containing 2 units and finally 0.2 cc of a 1:20 suspension of the antigen, the materials being added in this order. Following a period of 2 hours at room temperature to permit the fixation of complement, 0.4 cc of sensitized red cells were added, the tubes shaken and placed in a 37°C waterbath. Readings were taken at 15 minutes, 30 minutes, and 60 minutes, and a final one after the tubes had been permitted to stand at room temperature overnight. In practically all instances the significant changes had occurred at the end of 30 minutes. In every case anticomplementary controls were set up. None of the antigens showed anticomplementary effects even in 1:10 dilution (*i.e.*, twice the amount used in the actual test). Several sera had to be discarded, however, since non-specific fixation could be demonstrated when a 1 :4 dilution of the serum was used.

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[3] Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1923, **37**, 601.

[4] Kidd, J. G., *J. Exp. Med.*, 1940, **71**, 335 and 351.

[5] Kidd, J. G., *J. Bact.*, 1940, **39**, 349.

[6] Besredka, A., *Ann. Inst. Pasteur*, 1936, **56**, 125.

Results. The sera of 35 rabbits bearing tumors at the time of bleeding as proven by immediate autopsy were tested; of these 30 gave negative results. A number of the specimens of sera were run against more than one antigen, a total of 101 tests being recorded. Five sera definitely fixed complement on one occasion at least. When each was tested against several antigens the results shown in Table I were obtained.

The clinical state of the 5 rabbits furnishing positive sera is summarized in Table II.

TABLE I.
Antigens.

Serum	1	2	3	4	5	6	7	8	9	10	11	12	Positive	Negative	% Positive
201	+	0	0	0	—	0	—	0	—	+	+	—	3	5	37.5
352	+	+	+	+	—	0	—	—	—	—	0	+	5	2	71
361	0	0	+	0	—	0	—	—	—	—	0	—	1	5	17
473	—	+	+	0	0	0		—	—	—	0	—	3	4	43
498	0	0	+	0	0	0	0	—	0	—	0	—	1	8	11
													—	—	—
Totals													13	24	35

+ fixation of complement.
0 no fixation of complement.
— not tested.

Antigens	Rabbit No.	Tumor
1	103	frozen metastatic
2	107	glycerolated metastatic
3	107	” testicular
4	201	” ”
5	315	frozen metastatic
6	315	” testicular
7	327	” ”
8	400	” metastatic
9	400	” testicular
10	437	glycerolated testicular
11	471	” ”
12	473	frozen metastatic

TABLE II.

Rabbit	Days elapsed since inoculation	Clinical and pathological notes
201	31	Animal received multiple intratesticular, intraperitoneal and intramuscular inoculations. Animal beginning to lose weight. Tumors present at all inoculation sites plus widespread visceral and pulmonary metastases. Marked central necrosis in original growths.
352	36	Animal received bilateral intratesticular inoculations. In good condition at time of autopsy. Tumor limited to left testicle which showed advanced central necrosis with considerable surrounding inflammatory reaction.
361	35	Animal received bilateral intratesticular inoculations. Clinical condition poor with obvious weight loss and paralysis of hind quarters. Autopsy showed bilateral testicular involvement with the usual amount of central necrosis, and small omental and bilateral renal metastases.
473	26	Animal received bilateral intratesticular and intramuscular inoculations. Clinical condition poor with definite weight loss. Autopsy revealed typical take in left testes with many metastatic nodules of small size in the peritoneal cavity.
498	37	Animal received bilateral intratesticular inoculations. Clinical condition apparently good at time of being sacrificed. Autopsy showed bilateral testicular involvement with widespread abdominal and pulmonary metastases.

In the non-tumor bearing groups completely negative results were obtained throughout. The sera of 4 animals in whom clinical takes had recently regressed gave no evidence of complement fixation when tested against 3 known positive antigens. Specimens from 12 rabbits refractory to persistent intratesticular inoculations of tumor material failed to fix complement as did the sera of animals immunized by Besredka's method[6] (intracutaneous inoculation of the tumor followed by regression and the development of immunity against subsequent intratesticular inoculation of fresh neoplastic material).

Controls with normal sera were run with each antigen tested. Forty such tests were set up using specimens obtained from 16 rabbits uninoculated with the Brown-Pearce carcinoma. No specific fixation of complement was observed in any of the tests performed.

An attempt was made to ascertain whether the difficulty in securing positive results lay in the sera or in the antigens used. Dr. Kidd very kindly furnished 6 specimens of serum as follows:

Specimens 1, 2, 3 from domestic rabbits 5-01, 5-04, and 5-08 withdrawn before inoculation of tumor material.

Specimens 4, 5, 6 from domestic rabbits 5-01, 5-04, and 5-08,

TABLE III.

Serum	Positive reactions	Negative reactions
1	0	6
2	0	3
3	0	5
4	6	0
5	3	0
6	5	0

obtained 47, 54, and 47 days respectively after inoculation of the tumor. These 3 specimens had given consistently positive reactions in Dr. Kidd's hands. They were tried against the following antigens prepared by the author: Frozen metastases, 2 specimens; fresh metastases, 1 specimen; frozen testes, 3 specimens; glycerolated testes, 3 specimens.

The results are given in Table III.

It appeared, therefore, that the tumor extracts used in the present series of experiments did contain a substance which fixed complement in the presence of sera of rabbits bearing Brown-Pearce carcinoma growths used by Dr. Kidd in his original work.

Discussion. Among the sera of 35 rabbits bearing Brown-Pearce carcinoma in varying stages of progression and retrogression it has been possible to demonstrate the presence of complement fixing antibodies in only 5 specimens. Kidd's work has been confirmed, but the percentage of positively reacting sera is much lower in the present series than in that originally reported by him (14% as opposed to 80%). If it be assumed that no technical error invalidated the results of the test, the explanation for these divergent findings must be sought either in differences in the animals, in the time of bleeding, in the amount of tumor injected, or in the nature of the various antigenic preparations.

The animals used in the present series were a hybrid lot in contrast to the relatively more homogeneous group studied at the Rockefeller Institute. It is possible that the more frequent failure of the former group to develop antibodies is due to individual differences between the rabbits used.

Our animals were bled once—*i.e.,* just before being sacrificed. Since Kidd[4] has shown that rabbits may develop antibodies quite suddenly only to lose them rapidly within the course of 10 days, it is possible that a systematic bleeding at weekly intervals might have led to the discovery of more positively reacting sera.

Kidd's animals were given intramuscular as well as intratesticular inoculations. In nearly all cases those of the present series received only the latter. Possibly a greater antigenic stimulation resulted from Kidd's procedure. That this is an unlikely factor is suggested by observations that the amount and state of the tumor tissue appeared to bear no definite relation to the presence or absence of the specifically acting antibodies. Our data indicated that the presence of antibody was often associated with the existence of spreading metastases, although 15 rabbits with metastases failed to show the specific antibody.

In a personal communication to the author Kidd has stated that no difficulty had been experienced in securing suitable antigenic material from any specimen of Brown-Pearce carcinoma provided that the tissue was not entirely necrotic. The antigens used in the present series gave positive reactions with at least one serum (Rockefeller or Harvard) but the possibility of the existence of quantitative differences was suggested by the fact in no case did any of the author's sera give positive reactions with all the antigens (Table I). Thus the possibility exists that saline extracts of various tumors may vary in their capacity to fix complement in the presence of sera containing the specific antibody.

Summary. In confirmation of Kidd's work it has been demonstrated that a substance associated with the Brown-Pearce carcinoma fixes complement in the presence of serum of some rabbits bearing the specific tumor. But an incidence of positive reactors considerably lower than that reported by Kidd was observed. Several possible explanations for this discrepancy are discussed.

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Biological Properties of Pregneninolone (17-Ethinyl Testosterone) in Women.

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Inhoffen, Longemann and Serini[1] have synthesized a compound (Δ^4 pregnenin-20-on-3-ol-17; also known as pregneninolone, 17-ethinyl testosterone; anhydro-hydroxy-progesterone) which possesses an unusual variety of biological properties.

This compound, which is chemically very closely related to testosterone and progesterone, has been shown to have: (a) a progesterone-like action (progestomimetic) in immature rabbits being, furthermore, active when administered orally;[2, 3] (b) an estrogen-like action (estromimetic) on the uterus and vagina of adult and immature rats;[4, 5] and (c) an androgen-like action (andromimetic) in capons and rats.[4, 5, 6]

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[1] Inhoffen, H. H., Longemann, W., and Serini, A., *Ber. Deutsch. chem. Ges.*, 1938, **71**, 1024.

In humans, pregneninolone has been shown to produce a progestational effect on the estrogen-primed endometrium[7, 8] and to induce bleeding in cases of functional amenorrhea.[9] The study reported here was undertaken to determine if pregneninolone, administered to women, exhibited, in addition to the progestomimetic action, the same variety of biological properties (*viz.,* estromimetic and andromimetic) that it does in experimental animals.

Estromimetic. To determine whether pregneninolone had an estrogen-like action, a series of 20 post-menopausal and ovariectomized women, all of whom exhibited morphologic signs (by vaginal smears and endometrial and vaginal biopsies) of estrogen deficiency, were given doses of pregneninolone* (orally), varying from 30 to 60 mg per day, for periods varying from 35 to 56 days. The total doses administered varied from 2100 to 2940 mg. Vaginal smears were taken twice weekly; endometrial and/or vaginal biopsies at approximately 4-week intervals.

Results. No evidence of any estromimetic action was observed in the endometrium, vaginal mucosa or vaginal smears in the patients taking 30 mg per day. In 3 patients, taking 60 mg per day, slight estromimetic effects were noted in the vaginal smears after the ingestion of 3160, 3600 and 4200 mg of pregneninolone, in 8, 10 and 11 weeks, respectively. The smears in these cases revealed a scattering of large, squamous, epithelial cells among the "atrophy" cells. At no time, however, in spite of continued administration of the hormone, did the smears reveal a complete estrogenic effect. The endometrial biopsies revealed no evidence of a proliferative response.

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[2] Inhoffen, H. H., and Hohlweg, W., *Naturwissenschaften,* 1938, **26**, 96.

[3] Ruzicka, L., Hofmann, K., and Meldahl, H. F., *Helv. chem. Acta,* 1938, **21**, 372.

[4] Emmens, C. W., and Parkes, A. S., *Nature,* 1939, **143**, 1064.

[5] Salmon, U. J., and Salmon, A. A., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 709.

[6] Courrier, R., and Jost, A., *C. R. Soc. Biol. Paris,* 1939, **130**, 1162.

[7] Clauberg, C., and Ustum, Z., *Zentralbl. f. Gynakologie,* 1938, **62**, 1745.

[8] Salmon, U. J., Walter, R. I., and Geist, S. H., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 252.

[9] Zondek, B., and Rozin, S., *Lancet,* 1939, **1**, 504.

* For the pregneninolone used in this investigation, we are indebted to Dr. E. Schwenk, Schering Corporation, Bloomfield, N. J., and to Mr. R. C. Mautner, Ciba Pharmaceutical Products, Summit, N. J.

Andromimetic. To determine whether pregneninolone possessed androgen-like properties, the hormone was administered to a group of women with regular menstrual cycles. It has previously been shown that testosterone propionate, if administered to cyclical women (in adequate doses), causes: (a) suppression of menstruation;[10, 11] (b) regressive changes in the endometrium, resulting in hypoplasia or atrophy;[10, 12, 13] (c) regressive changes in the vaginal mucosa, manifested by the appearance of estrogen deficiency smears;[11, 13, 14] and (d) masculinization (arrhenomimetic) phenomena (coarsening of the voice and lowering of its pitch; facial hirsuties; enlargement of the clitoris).[10, 13, 15]

Sixteen patients were given pregneninolone in doses varying from 30 to 60 mg per day, for periods varying from 3 to 20 months. The total amounts administered varied from 760 to 10,480 mg.

Results. In no instance were the menstrual cycles significantly affected. The vaginal smears did not reveal any signs of regression in any of the cases. None of the patients developed any of the arrhenomimetic phenomena. It is interesting to note, furthermore, that none of the patients developed acne—a not infrequent result of the administration (by the intramuscular route) of 500 mg or more of testosterone propionate.

Summary and Conclusions. Pregneninolone, though chemically very closely related to both testosterone and progesterone, does not manifest in women the same multiplicity of biological properties that it does in experimental animals. In women, its most striking property is its progestomimetic effect on the estrogen-primed endometrium, when administered orally.

In contrast to its action in rats, in women pregneninolone appears to have: (a) no estromimetic effect on the endometrium and only a very slight effect on the vaginal epithelium if given in very large doses; and (b) no arrhenomimetic effects.

Two interesting observations emerge from this study that are worthy of note. First, the profound change in biologic activity that resulted from the introduction of the ethinyl group at the 17th

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[10] Geist, S. H., Salmon, U. J., and Gaines, J. A., *Endocrinology*, 1938, **23**, 784.

[11] Papanicolaou, G. N., Ripley, H. S., and Shorr, E., *Endocrinology*, 1939, **24**, 339.

[12] Gaines, J. A., Salmon, U. J., and Geist, S. H., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 779.

[13] Salmon, U. J., Geist, S. H., and Walter, R. I., *Am. J. Obs. and Gyn.*, 1939, **38**, 264.

[14] Salmon, U. J., Walter, R. I., and Geist, S. H., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 467.

[15] Geist, S. H., Salmon, U. J., Gaines, J. A., and Walter, R. I., *J. A. M. A.*, 1940, **114**, 1539.

carbon atom of the testosterone molecule, which is the only manner in which pregneninolone differs from testosterone. Second, the striking difference in the biological properties displayed by the same compound when administered to experimental animals and to humans.

The results of this study draw attention to the inadvisability of applying to humans, observations concerning the biological properties of hormones as determined in animal experiments and emphasizes the importance of determining (by means of objective studies) the biological properties of these compounds in humans before using them as therapeutic agents.

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Thermostable Oxidations in Tumor and Muscle Tissue.

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Hopkins and Dixon[1] showed that if a tissue was minced, thoroughly extracted with boiling water, dehydrated with alcohol, dried and finely ground, the resulting "thermostable powder" would not take up oxygen by itself, but in the presence of a little glutathione would take up considerable amounts of oxygen. Further work by Hopkins[2] indicated that the oxygen uptake of these thermostable powders was due to the oxidation of fat at an acid pH and the oxidation of protein at neutral or slightly alkaline pH.

We were interested in determining whether tumor tissue possessed any thermostable oxidative properties similar to those Hopkins found for muscle tissue. For our experiments we have used Flexner-Jobling rat carcinoma and have compared all results to those obtained with corresponding thermostable powders prepared from normal rat muscle. Muscle was selected as the comparison tissue principally because the aerobic respiration of fresh minced tissue of the Flexner-Jobling rat carcinoma is of the same order of magnitude as the aerobic respiration of fresh rat skeletal muscle mince.

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* This report is from a thesis submitted by Robert N. Feinstein to the Graduate School of the University of Wisconsin in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

† Now at the School of Medicine, University of Chicago, Chicago, Illinois.

A preliminary report of this work was presented before the thirty-third annual meeting of the Federation of American Societies for Experimental Biology in Toronto, April, 1939.

[1] Hopkins, F. G., and Dixon, M., *J. Biol. Chem.*, 1922, **54**, 527.

[2] Hopkins, F. G., *Biochem. J.*, 1925, **19**, 787.

Methods. The powders were prepared by mincing the tissue in a Latapie mincer, suspending them in 3 volumes of distilled water, boiling for 10 minutes, decanting the supernatant fluid, and resuspending in boiling water. The powders were then washed with hot water by decantation 9 times. From the time the tissue was first suspended in water, anaerobic conditions were maintained by bubbling a stream of nitrogen through the solution. After the last decantation, the tissue was washed twice with 95% alcohol and finally with absolute alcohol. The resulting material was dried *in vacuo* at a temperature of 60°C. When dry, it was thoroughly ground and stored in an evacuated Thunberg tube in the ice box. For some experiments, the powders were extracted with chloroform for 24 hours in Soxhlet extractors, and the resulting fat-free powders were used. When the powders were thus extracted, it was found most important to remove all traces of the chloroform, for the barest trace of any volatile, water-insoluble solvent can introduce considerable error in manometric experiments.

The glutathione used was a crystalline preparation of reduced glutathione. In the presence of a little copper, it took up oxygen equivalent to the theoretical amount as calculated from the equation:

$$4\ GSH + O_2 \rightarrow 2\ GSSG + 2\ H_2O$$

The measurements of oxygen uptake were made in the Warburg manometric apparatus at a temperature of 38.2°C, and oxygen uptakes were calculated on the basis of cu mm of oxygen per gram of powder. 0.15 g of the thermostable powder was weighed into the flask; then the desired amount of glutathione, usually 3 mg, was added. As the flasks were calibrated to contain 2.2 cc, the concentration of glutathione was M/200. The medium used was an unbuffered Ringer's solution brought to the desired pH with HCl or NaOH. Such a Ringer's solution is satisfactory for the respiration medium, since no acid is produced. CO_2 production was found to be negligible, so no KOH was used in the flasks. When any substance, such as $CuSO_4$, iodoacetate, etc., was to be added, it was dissolved in Ringer's solution and brought to the proper pH.

Chemical determinations were run on the fat-free powders. Total nitrogen was determined by Kjeldahl digestion with a $CuSO_4$-K_2SO_4-H_2SO_4 mixture. Total sulfur and total phosphorus were determined on a single sample, by sodium peroxide oxidation in a Parr sulfur bomb. The sulfur was precipitated as $BaSO_4$ and determined gravimetrically, while the phosphorus was precipitated from the filtrate as the strychnine phosphomolybdate.[3] This determination of phosphorus compared satisfactorily with the H_2SO_4-HNO_3-$HClO_4$ digestion method of Gerritz.[4] The sulfhydryl content of the powders was determined by the method of Todrick and Walker,[5] measuring the reduction of 2,6-dichlorophenolindophenol in a boiling water bath. To prevent recoloration of the dye the determinations were carried out in test tubes bearing one hole rubber stoppers and filled with nitrogen before placing in the bath. Since the dye was not calibrated against pure cysteine, but was simply weighed out and made to volume, no accuracy is claimed as to the *absolute* figures for -SH content, but the striking *differences* in -SH content observed are significant. Determinations of -SH content of the tumor and the muscle powder were run alternately, during the course of a single hour, and with the same solution of the dye, so that exactly the same titer of the dye was used for both.

Results. Although fresh minced tissue of the Flexner-Jobling rat carcinoma has an aerobic respiration of the same order of magnitude as fresh rat skeletal muscle mince, the thermostable oxidative properties are markedly lower in the tumor powder than in the muscle. A summary of some typical results is given in Table I.

From Table I it is observed that fresh minced tumor and muscle tissue consume approximately equal amounts of oxygen, namely 2600 and 2300 cu mm per hour per gram, respectively. Under optimal conditions, however, the thermostable powder prepared from tumor tissue consumes only 167 cu mm, while the muscle powder takes up 464 cu mm.

TABLE I.

Oxygen Uptake of Fresh Tissue and of Thermostable Powders Prepared from Fresh Tissues.

	Tumor	Muscle
	cu mm O_2 per g dry wt per hr	
Fresh Tissue (mince)	2600	2300
Thermostable Powder	32	104
” ” + M/1000 $CuSO_4$	60	176
” ” + M/200 glutathione	85	175
” ” + M/1000 $CuSO_4$ M/200 glutathione	167	464
” ” + M/100 NaCN	40	7
” ” + M/200 glutathione M/100 NaCN	36	7

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[3] Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry*, Vol. II, p. 873, Williams and Wilkins, Baltimore, 1932.

[4] Gerritz, H. W., *Ind. Eng. Chem., Anal. Ed.*, 1935, **7**, 116.

[5] Todrick, A., and Walker, E., *Biochem. J.*, 1937, **31**, 292.

Table I shows also that both copper and glutathione are necessary for maximum oxygen uptake. For the oxidation 2 GSH ⟶ GSSG, approximately 60 cu mm of oxygen are taken up by the 3 mg of glutathione used in this experiment. It is of interest that under the best conditions, the thermostable oxygen uptake of the muscle powder is equivalent to about 20% of the oxygen uptake of fresh muscle tissue; the thermostable oxygen uptake of the tumor powder is only 6% of the oxygen uptake of the fresh tissue. The cyanide inhibition of the thermostable oxygen uptake is markedly different; with the tumor powder an inhibition of 50% was observed, and with the muscle powder an inhibition of 95%.

In the thermostable powders, protein is probably the most important substrate. This view is suggested by several facts. First, the source and preparation of the powders are such that protein is the main constituent. Second, under similar conditions, pure native or denatured egg albumin, plus a little glutathione and copper, takes up oxygen. Third, if the thermostable powders, either normal or tumor, are extracted for 24 hours with chloroform, the oxygen uptake of the resulting fat-free powders is not decreased, but may actually be increased somewhat, as though the powders were losing so much inert material. Fourth, the powders, either fat-containing or fat-free, take up approximately the same amounts of oxygen at acid or neutral pH.

Table II gives the results of the chemical determinations. The total phosphorus is appreciably greater in the tumor powder than in the muscle powder. The nitrogen and the total sulfur content are not significantly different in the two powders. However, it appears of significance that the sulfhydryl content of the tumor powder is only 1/7 that of the muscle powder. Since the glutathione has been removed from these powders by the washing during their preparation, any sulfhydryl found is probably from the denatured protein.

The conclusion drawn by Hopkins[2] as to the importance of the protein -SH group in this oxidation seems to be confirmed by the

TABLE II.
Chemical Comparison of Thermostable Powders Prepared from Fresh Tumor and Muscle Tissue.

	Lipid-free tumor powder	Lipid-free muscle powder
Nitrogen	11.90%	13.80%
Total Phosphorus	0.74	0.11
" Sulfur (calculated as S)	1.16	1.14
" " (" " cysteine)	4.40	4.30
Sulfhydryl Sulfur (as cysteine)	0.07	0.51
% of total Sulfur present as Sulfhydryl	1.60	11.90

fact that the oxidation is inhibited by the presence of iodoacetate, especially after prolonged incubation, either aerobic or anaerobic. Iodoacetate is believed to exert its inhibitory effects by combination with active thiol groups.[6-10]

Summary. It has been shown that although minced Flexner-Jobling rat carcinoma has an aerobic respiration equal to that of rat skeletal muscle mince, the oxygen uptake in the presence of glutathione of thermostable powders (essentially denatured proteins) prepared from the tumor tissue is considerably lower than the oxygen uptake of similar powders prepared from muscle. This seems to parallel the fact that although the tumor powder and the muscle powder have approximately the same total sulfur content, the percent of the total sulfur which is present as sulfhydryl sulfur is much lower in the tumor powder than in the muscle powder.

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Oxidation of Native Protein by Glutathione-Copper Catalysis.

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That protein can, under certain conditions, consume oxygen has been known for some time. In explanation of this phenomenon 3 factors have been stressed: (1) heavy metals, (2) a low molecular weight source of reversibly oxidizable sulfur such as cysteine or glutathione, and (3) the content and chemical state of the sulfur of the protein substrate.

Our work has been entirely with native egg albumin and gelatin, as examples of proteins of high and low sulfur content respectively. We were interested in studying the effects of variations in the three

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[6] Dickens, F., *Biochem. J.*, 1933, **27**, 1141.

[7] Dickens, F., *Nature*, 1933, **131**, 130.

[8] Rapkine, L., *Compt. rend.*, 1933, **112**, 790.

[9] Rapkine, L., *Compt. rend.*, 1933, **112**, 1294.

[10] Rapkine, L., *J. chim. phys.*, 1936, **33**, 493.

* This report is from a thesis submitted by Robert N. Feinstein to the Graduate School of the University of Wisconsin in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

† Now at the School of Medicine, University of Chicago, Chicago, Illinois.

TABLE I.
Effect of Cu, Fe, and Glutathione (GSH) on Oxygen Consumption of Native Egg Albumin.

g of albumin	Molarity of GSH	Molarity of $FeCl_3$	Molarity of $CuSO_4$	cu mm O_2 used, 6 hr
.15	M/200	M/1000	0	30
.15	M/200	0	M/1000	62
.15	M/200	M/1000	M/1000	57

factors listed above. Measurements of oxygen uptake were made in the Warburg manometric apparatus at a temperature of 38.2°C. The protein was weighed directly into the flasks, and the other materials were dissolved in unbuffered Ringer's solution brought to pH 7.4 and pipetted in. The final volume of the flask contents was 2.2 cc.

Results. In Table I are listed the results of variations in heavy metal. If either glutathione (GSH) or all heavy metals are absent, no oxygen is consumed. If the protein is omitted, the oxidation of the GSH is so rapid as not to be measured by the technic employed. It is of particular interest that gelatin, under the same conditions, consumes approximately the same quantity of oxygen, despite the wide disparity in sulfur content of the two proteins.

Another point of interest is the difference in behavior of egg albumin and gelatin to dilute and concentrated solutions of $CuSO_4$. The data are presented in Table II, which shows that gelatin is inhibited in its oxygen consumption about 20% by concentrated solutions of $CuSO_4$, while the egg albumin is not inhibited but somewhat increased. Not only is the rate of oxygen uptake increased by the M/10 $CuSO_4$ in the latter case, but also the albumin consumes considerably more oxygen before the reaction ceases.

Part of this excess oxygen uptake is independent of the presence of the GSH. In one experiment, 0.15 g of egg albumin in the presence of M/200 glutathione and M/10 $CuSO_4$ consumed 77 cu mm of oxygen in 6 hours, while 0.15 g of the protein in M/10 $CuSO_4$ with no added glutathione still used 56 cu mm (both reactions still well in

TABLE II.
Difference in Effect of Concentrated and Dilute $CuSO_4$ on Gelatin and Egg Albumin.

Protein	Molarity of $CuSO_4$	cu mm O_2 used, 4 hr
Gelatin	.002 M	81
”	.1 M	64
Egg Albumin	.002 M	76
” ”	.1 M	85

GSH = 3 mg (M/200); Protein = .15 g.

progress). This GSH-independent oxygen consumption is possibly due to denaturation of the protein by the copper, with consequent exposure and oxidation of latent -SH groups. Gelatin, with its low sulfur content, is not similarly affected.

Summary. Native egg albumin and gelatin, in the presence of M/1000 $CuSO_4$ and M/200 glutathione, consume significant and approximately equal quantities of oxygen. When the $CuSO_4$ is replaced by M/1000 $FeCl_3$, the oxygen uptake is diminished by about 50%. If the M/1000 $CuSO_4$ is replaced by M/10 $CuSO_4$, the gelatin consumes less oxygen than before, and the egg albumin more.

11741

Calcification of Trichina Cysts *in vitro*.*

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Previous attempts to produce pathological calcification *in vitro*[1, 2] have failed to give consistent results, presumably owing to failure to employ a readily calcifiable tissue. Because trichina cysts become calcified in a uniform manner *in vivo,* we attempted to reproduce this phenomenon *in vitro,* employing the technics used for rachitic cartilage.[3] Preliminary experiments failed to produce calcification even when serum of high $Ca^{++} \times P$ product was used. In view of the findings of von Brand, Holtz and Vogel[4] and of Wantland[5] in which it was demonstrated that large doses of viosterol greatly accelerate the calcification of the cysts *in vivo,* calcification *in vitro* was attempted in infected muscle from rats which had received one or more toxic doses of viosterol. The experiments demonstrated that following this preliminary treatment, calcification of the cysts *in vitro* could be readily obtained.

Methods. Adult rats of varying strains, ages and weights, which

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* This work was aided by a grant from the Josiah Macy, Jr., Foundation. We are indebted to Mead Johnson and Co. for supplies of viosterol, 1,000,000 units per gram.

[1] Rosenheim, A. H., and Robison, R., *Biochem. J.*, 1934, **28**, 712.

[2] Law, K. A. O., and Robison, R., *Biochem. J.*, 1936, **30**, 69.

[3] Shipley, P. G., Kramer, B., and Howland, J., *Biochem. J.*, 1926, **20**, 379.

[4] v. Brand, T., Holtz, F., and Vogel, H., *Z. f. Parasitenk.*, 1933, **6**, 308.

[5] Wantland, W. W., *J. Parasitol.*, 1938, **24**, 167.

had been infected with *Trichinella spiralis* 18 to 277 days before their tissues were incubated, were used. They were fed viosterol for from 1 to 5.5 days, the object being to predispose the cysts to calcification, but to avoid extensive calcification *in vivo*. The rats were then killed, and their diaphragms were removed aseptically and cut into pieces about 10 mm square. One piece from each diaphragm was fixed in 10% neutral formalin, as an unincubated control, and other pieces were placed in the incubating medium, which in each case was fresh sterile dog serum. In each experiment the Ca^{++} x P product was elevated in one-half the flasks by the addition of 0.1 cc of M/10 sodium phosphate mixture (20% NaH_2PO_4 + 80% Na_2HPO_4; pH 7.35) to 5 cc of serum. Incubation was carried out in tightly stoppered 50 cc Erlenmeyer flasks. No attempt was made to replace the lost carbon dioxide. Phenol red was added to each flask in order to observe any increased acidity due to bacterial contamination. The pH was not determined for individual flasks, but in representative samples was found to average about 7.5.

After incubating at 37°C for 24 hours, and for varying periods thereafter, the tissues were fixed in 10% neutral formalin, stained for calcium phosphate by the von Kóssa method, dehydrated with acetone, and cleared with isosafrol. In some cases they were also embedded in nitrocellulose, sectioned, stained by the von Kóssa method, and counterstained with hematoxylin and eosin or with eosin alone. The serum used was in each case analyzed for total calcium, phosphate, and protein, and the Ca^{++} concentration was estimated from total calcium and total protein.[6]

Results. No calcification of the cysts, either before or after incubation, was observed in the tissues of any of the animals which had not received viosterol, either with or without the addition of phosphate to the incubating medium. Tissues from 6 rats were so incubated, at from 48 to 229 days after infection. Calcification of the interstitial tissue occurred to a slight extent during incubation, as did calcification of the cut ends of the muscle. The cysts were free of any calcium deposits, and were, if anything, refractory.

Calcification of the cyst capsule *in vitro* was observed in tissues obtained from rats which had been infected for 35 days or more and which had received viosterol either as 150,000 units daily for at least 4 days or as 1,000,000 units in one dose at least 3.25 days previously. In the majority of instances this treatment initiated calcification *in vivo,* the amount of calcification being markedly increased by incubation with serum. The results in the tissues from

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[6] McLean, F. C., and Hastings, A. B., *J. Biol. Chem.*, 1935, **108**, 285.

TABLE I.

Calcification of Trichina Cysts *in vitro* Following Administration of Viosterol to Infected Rats.

Rat No.	Age of infection (days)	Viosterol (USP XI units)	No. of doses	Days between first dose and incubation	$Ca^{++} \times P$ moles $\times 10^6$	Calcification of cysts 0	24	48	72 hr
36	251	150,000 daily	2	2	1.73	0	0	0	0
					4.59	0	0	0	0
35	269	150,000 daily	5½	5.5	1.95	++++	++++	++++	++++
					4.62	++++	++++	++++	++++
33	281	150,000 daily	4	4	1.85	++	++	++++	++++
					4.55	++	+++	++++	++++
					3.04	++	+++	++++	++++
40	110	150,000 daily	4	4	1.37	±	+++	++	
					4.18	±	++++	+++	
46	138	1,000,000	1	4	1.77	++++	++++		
					4.71	++++	++++		
47	138	1,000,000	1	3	1.77	±	++		
					4.71	±	+		
48	138	1,000,000	1	2	1.77	+	+		
					4.71	+	0		
49	138	1,000,000	1	1	1.77	±	0		
					4.71	±	±		
51	159	150,000 daily	4	4	1.45	+	++	++	
					3.99	+	+++	+++	
52	159	1,000,000	1	3.5	1.45	++	++++	++++	
					3.99	++	++++	++++	
53	159	1,000,000	1	3.25	1.45	±	+	++	
					3.99				
60	183 *18	1,000,000	1	4	1.76	0	0	0	
					4.64	0	0	0	
61	183 *18	1,000,000	1	3.5	1.76	+	+		
					4.64	+	+++	++++	
62	18	1,000,000	1	4	1.76	0	0	0	
					4.64	0	0	0	
63	18	1,000,000	1	3.5	1.76	0	0	0	
					4.64	0	0	0	
65	207 *35	1,000,000	1	4	1.47	+	++	+**	
					4.06	+	++	+++	
66	35	1,000,000	1	4	1.47	0	±	+**	
					4.06	0	+	+	
67	207 *35	1,000,000	1	3.5	1.47	+	+++	++**	
					4.06	+	++++	++++	
68	35	1,000,000	1	3.5	1.47	0	0	±**	
					4.06	0	±	0	

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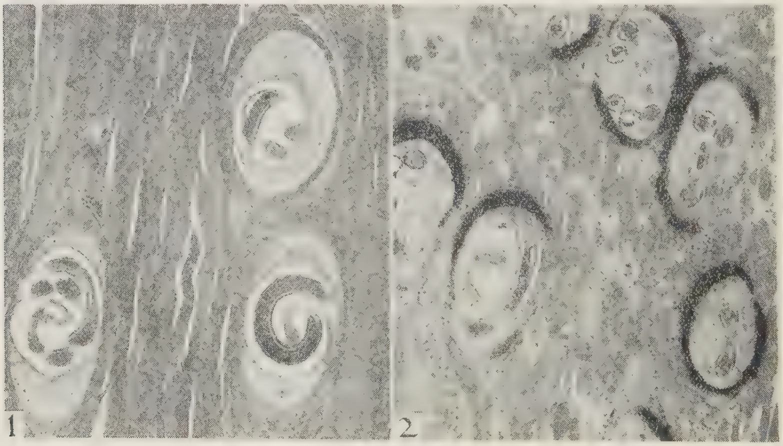
*Superinfection.

**Evidence of lowered pH owing to bacterial contamination.

animals treated with viosterol are summarized in Table I. Because the total calcium, total protein, and Ca^{++} concentrations varied but little, and because marked variation in the concentration of phosphate occurred only with the addition of phosphate to the medium, the extended data for these analyses are not given. Table I includes the figures for $Ca^{++} \times$ total P, when both are expressed in mols per kilogram of water.

The calcification process observed in the incubated tissues was similar to that observed *in vivo,* being localized in the capsules of the cysts, usually starting at the poles and involving the entire capsule as the process progressed (Fig. 2). This was usually accompanied by a small amount of interstitial calcification not exceeding that observed in tissues from animals not treated with viosterol. There was some tendency for calcifiability of the cysts to increase with their age.

Calcification of the cysts, when it occurred, was not found to be limited by the same $Ca^{++} \times P$ product found for the calcification of rachitic cartilage *in vitro.*[3, 7] In these experiments calcification occurred uniformly with sera having products from 1.37 to 1.95×10^{-6}, and when phosphate was added to raise the product above 3.0×10^{-6}, the critical product for the calcification of rachitic cartilage, the in-



FIGS. 1 AND 2.

Sections through diaphragm of rat No. 40, 110 days after infection with *Trichinella spiralis.* Silver nitrate-eosin. Photomicrographs, × 80. Fig. 1. Unincubated control. Whole mounts demonstrated minimal calcification of few cysts, not seen in sections. Fig. 2. After incubation for 48 hours in serum to which phosphate had been added. Heavy calcification of capsules of cysts.

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[7] Robison, R., and Soames, K., *Biochem. J.*, 1930, **24**, 1922.

tensity of calcification was increased only slightly, if at all (Table I).

In an attempt to shed some light on the nature of the local changes associated with calcification, sections of infected muscles were stained for phosphatase by a modification of the method of Gomori.[8] Using this method, phosphatase was found to be present in all cysts which were old enough to be calcified following the use of viosterol. Its location was delimited to the region within the capsule, adjacent to the worm. In this respect it differed from the localization of calcification, for the latter characteristically occurred within the capsule substance, and not in the space confined by the capsule. The enzyme was present before any viosterol was administered, and such treatment did not visibly change its concentration or its distribution.

Discussion. The experiments reported were successful in producing a form of pathological calcification *in vitro,* the localization of the calcification and the conditions for its occurrence being predictable. Material is thus available for the further study of controlled pathological calcification. The fact that this calcification occurs at $Ca^{++} \times P$ products below those necessary for calcification of rachitic cartilage matrix indicates that the mechanisms of the two forms of calcification are not identical. This is consistent with the occurrence of various forms of pathological calcification in adults whose serum does not contain calcium and phosphorus in concentrations sufficient to produce calcification of rachitic cartilage.

In so far as the mode of action of viosterol is concerned, the experiments indicate that the action in this case is something more than to increase the absorption of calcium and of phosphate, and thus to raise the $Ca^{++} \times P$ product in the plasma. But it must be emphasized that the dosage employed was in the toxic range, and that the results do not necessarily bear any relation to the prophylactic and therapeutic actions of vitamin D in small doses. Instead it seems probable that the action is related to the known toxic actions of viosterol, and that the predisposition of the trichina cysts to calcification *in vitro* is the result of a toxic action upon the worm or its cysts, just as the soft tissues of the organism may be predisposed to calcification *in vivo* by the same action.

Demonstration of phosphatase in the cysts adds another instance to those in which this enzyme is found in tissues subject to calcification. In this instance, however, it is present in the cysts for some months before calcification usually occurs spontaneously in rats, and it has not been possible to demonstrate any change in its concentration or distribution under a procedure which predisposes the cysts to calcification.

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[8] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 23.

Summary. 1. Trichina cysts did not calcify spontaneously *in vitro,* even when exposed to media with high $Ca^{++} \times P$ products. 2. When calcification was initiated *in vivo,* by administration of toxic doses of viosterol over a period of several days, incubation with serum *in vitro* led to a marked increase in the amount of calcification of the cysts. 3. Calcification of the cysts, when it occurred at all, occurred at $Ca^{++} \times P$ products well below the critical point for the calcification of rachitic cartilage. 4. The action of viosterol in predisposing to calcification, both *in vivo* and *in vitro,* is attributed to a toxic effect upon the cyst or the worm. 5. An enzyme which hydrolyzes glycerophosphate is present in trichina cysts. It is present in the absence of administration of viosterol, which did not affect either its concentration or its distribution. It is present in the cysts for several months before calcification usually occurs spontaneously *in vivo* in rats.

11742 P

Tissue Response to p-Amino Benzene Sulfonamide (Sulfanilamide) in Mice.

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With the greatly increasing use of the sulfonamide compounds in medicine in the past several years, a lively interest has arisen in regard to the effects of these drugs on the organism outside of their now well known activity in combating various types of infections. Numerous toxicological studies have therefore appeared on this subject. The somewhat equivocal relationship of p-amino benzene sulfonamide (sulfanilamide) to some of the aniline dyes suggested the possibility that the former might have mild carcinogenic properties similar to those reported for the aniline dyes,[1] and prompted the investigation described below. Subsequently, it came to the author's attention that a similar investigation had already been carried out by Lewis,[2] at the suggestion of Dr. E. K. Marshall. The results were subsequently confirmed by Zamecnik and Koletsky.[3] These workers used pedigreed mice that were known to have a natural genetic resistance to neoplasms. Olive oil was the suspending medium used in both these investigations. Both reported that sulfanilamide was carcinogenically inert under these conditions. The following experiment was carried out under slightly differently controlled conditions and the preliminary results are herewith presented because they are at variance with those reported by the authors mentioned.

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* Assistant, Department of Medicine, University of Chicago.

[1] Hueper, W. C., Wiley, F. H., and Wolfe, E. J., *J. Ind. Hygiene,* 1938, **20**, 46.

[2] Lewis, M. R., *Am. J. Cancer,* 1938, **34**, 431.

[3] Zamecnik, P. C., and Koletsky, S., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 391.

One hundred Swiss Albino mice were obtained from the Roscoe B. Jackson laboratory in Bar Harbor, Maine. These animals are not pedigreed but the natural incidence of cancer is low. An attempt was made to compare the action of sulfanilamide with 1:2:5:6 dibenzanthracene, a known carcinogenic compound in these animals. Lard was used as the suspending agent to insure slow absorption. Identical adequate food and living conditions were maintained. Feeding experiments were also undertaken. Merck's sulfanilamide and Eastman Kodak 1:2:5:6 dibenzanthracene were used. The experiment was arranged as follows:

Group I. Twenty mice were injected on 2 occasions with 15 mg dibenzanthracene in lard. They received a regular diet. Eight of these animals (40%) developed spindle cell sarcomas at the site of injection and died in 150 to 200 days after the first injection.

Group II. Twenty mice were injected with 15 mg sulfanilamide in lard on 2 occasions. They received a regular diet. Two animals (10%) developed tumors at the site of injection and died at 222 and 261 days respectively. Six animals are still alive. The tumors were frankly invasive and at death grossly deformed the animal. In one instance the tumor invaded the muscles of the leg and in the other instance the tumor eroded through the body wall, ribs and at autopsy was observed to be densely adherent to the liver. Microscopic section revealed the tumors to be composed predominantly of spindle cells with many polymorphic and tumor giant cells. The tumors were not transplanted. This procedure is contemplated, however. No metastases were seen but tumor cells were seen invading the liver in the instance mentioned above. Numerous mitotic figures were seen.

Group III. Twenty mice were injected with 15 mg dibenzanthracene in lard on 2 occasions. They received a diet which contained 2½% sulfanilamide. All the animals died prior to 150 days. Two spindle cell sarcomas were noted at the injection site (10%) at this time.

Group IV. Twenty mice were simply fed a diet containing 2½%

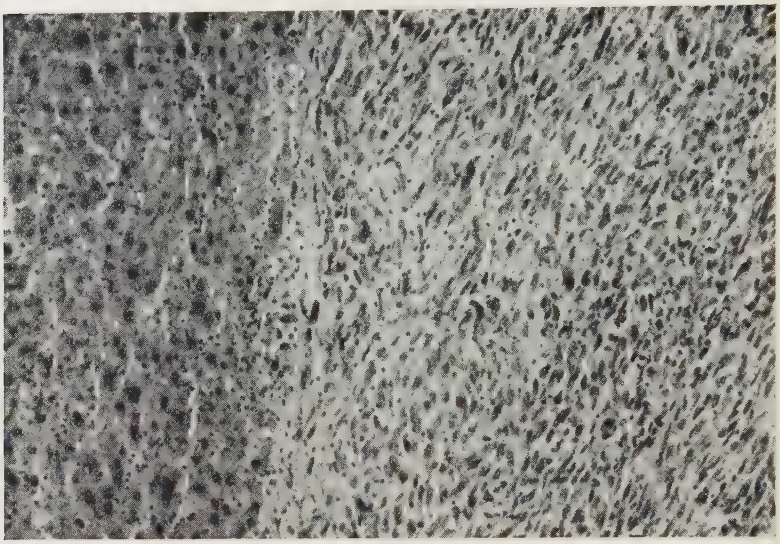

FIG. 1.

sulfanilamide. No injections were made. Most of the group is still alive. No tumors have been noted.

Group V. Twenty mice were fed a regular diet. No injections were given. Most of the group is still alive. No tumors have been noted.

In addition to the wide experience of numerous workers in the field of carcinogenic investigation which has pretty well established the fact that lard itself is not carcinogenic in numerous strains of mice,[4] the author cites the experience of Dr. A. Brunschwig who has been working with the above mentioned strain of mice in this laboratory at the same time as the author. The former worker obtained no tumors in Swiss albino mice following the injection of lard alone. This series contained 120 animals.[5]

Several additional observations were made in these experiments which will be reported at a later date. This work is being repeated and extended and a similar experiment is being carried out with sulfapyridine.

Summary. The subcutaneous injection of Merck's sulfanilamide suspended in lard into 20 Swiss Albino mice was followed in 2 instances by the development of a spindle cell sarcoma at the site of injection. The animals died at 222 and 261 days respectively. A similar group of mice treated similarly with 1:2:5:6 dibenzanthracene resulted in spindle cell sarcomas at the site of injection in 8 animals. The latter died in 150 to 200 days.

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[4] Andervont, H. B., *Public Health Lib., U. S. Treas. Dept.,* 1934, **49**, 620.

[5] Personal communication.

11743

Effect of Elementary Phosphorus Upon the Epiphysis of the Albino Rat.*

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(Introduced by L. Dienes.)

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Wegner described the development of a sclerotic zone in the vicinity of growing epiphyseal cartilage following the administration of small amounts of elementary phosphorus.[1] Since the process was subsequently found to be opaque to roentgen rays[2, 3, 4] it occurred to one of us (J.S.B.) that this phosphorus line might offer an *in vivo* radiographic marker of the varied growth rate in the epiphyseal region. No clear conception of the developmental mechanism of the line was available and this study was therefore performed in order to investigate its histogenesis.

Material and Methods. Subjects were albino rats 4 to 8 months of age sustained upon the BMS regimen recommended by Russell.[5] There were 28 experimental animals, 6 of which were utilized as controls. To the remainder a daily dose of 2.0 cc of elementary phosphorus in oil of peach kernels was administered by gavage. The solution contained 0.05 mg of elementary yellow phosphorus per cubic centimeter. Roentgenograms were taken of the limbs at intervals in order to detect the development of the opaque area of osteosclerosis.

Animals were sacrificed at periods up to 68 days after the initial administration of phosphorus. Both femurs and tibias were sub-

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* The expenses of this study were defrayed by a grant from the Infantile Paralysis Research Fund of the National Foundation for Infantile Paralysis, Inc.

[1] Wegner, G., *Virch. Arch.*, 1872, **55**, 11.

[2] Park, E. A., Jackson, D., Goodwin, T. C., and Kadji, L., *J. Ped.*, 1933, **3**, 265.

[3] Phemister, D. B., *J. A. M. A.*, 1918, **70**, 1737.

[4] Phemister, D. B., Miller, E. M., and Bonar, B. E., *J. A. M. A.*, 1921, **76**, 850.

[5] Russell, W. C., *J. Nutrition*, 1932, **5**, 347.

jected to investigation. Intact tibias were fixed in 90% alcohol and using the Ingalls modification of the Gomorri silver stain[6] the degree of mineralization in the epiphyseal region was determined. The lower end of each femur was split longitudinally, fixed in Zenker's fluid with 5% acetic acid, sectioned in paraffin, and stained with phloxine-methylene blue.

In the normal rat, calcification occurs in that portion of the epiphyseal cartilage adjacent to the metaphysis.[7] Erosion of the lacunae and removal of effete chondroblasts leaves only thin longitudinal strands of calcified matrix constituting the primary trabeculae. Physiologic factors thereupon cause the resorption of the great majority of these, and the remainder, following the deposition of bone, comprise the secondary or true metaphyseal trabeculae. (Fig. 2.) Among the marrow constituents (osteoblasts, hematopoietic elements, etc.) an occasional multinucleated osteoclast is apparent.

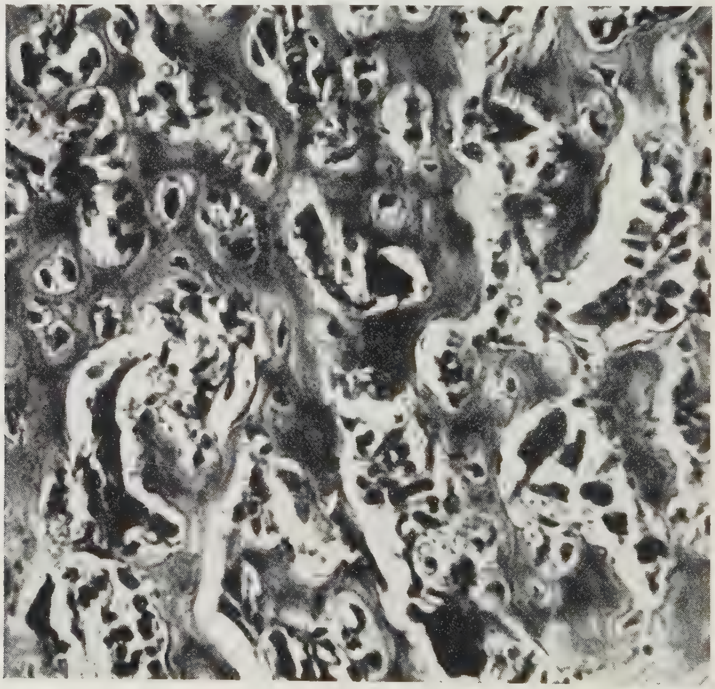

FIG. 1.
The appearance of the metaphyseal marrow seven days after the administration of elementary phosphorus. Seven large dark staining irregular osteoclasts are evident. × 400.

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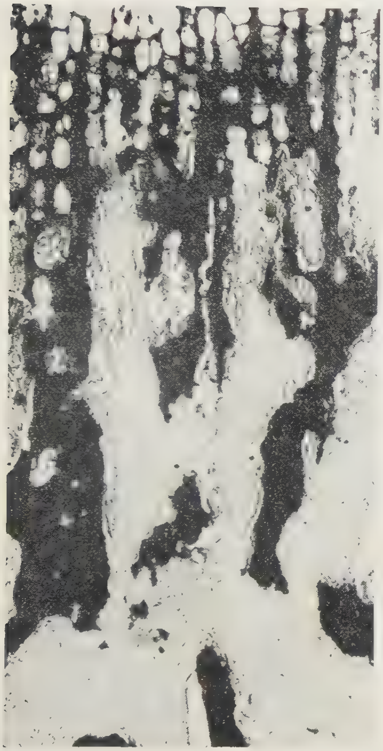
[6] Ingalls, T. H., and Merrill, O. E., *J. Nutrition*, in press.

[7] Dodds, G. S., and Cameron, H. C., *Am. J. Anat.*, 1934, **55**, 135.



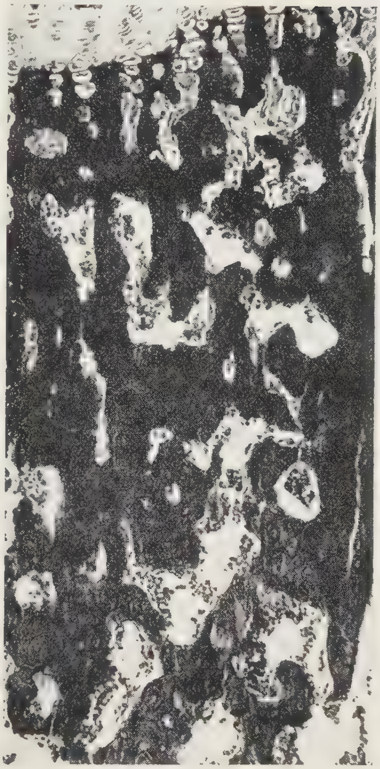


FIG. 2 (left).

A silver stain of the subepiphyseal region in the tibia of a normal albino rat. Mineralized cartilage and bone stain black. Primary spicules are much more numerous than the secondary trabeculæ. × 150.

FIG. 3 (right).

A similar area in the tibia of a rat 28 days after the initial administration of elementary phosphorus. Primary trabeculæ are not resorbed and the number of secondary trabeculæ is therefore increased. × 150.

Within 3 to 9 days after the institution of the experimental phosphorus regime there was an increase in the number of osteoclasts present, particularly in the marrow immediately proximate to the primary trabeculae. (Fig. 1.) This increase was progressive and simultaneously it became evident that the area of primary ossification contained more trabeculae than were normally present. The appearance of individual trabeculae was unchanged but it was apparent that there were many more persisting through to the stage of ossification. The secondary trabeculae were not only more numerous than those in the controls but they were also considerably longer. Metaphyseal cancellous bone extended for a distance of 2 or 3 mm into the shaft instead of a fraction of a millimeter as was normally the case. Intervening marrow content was lessened in amount as a concomitant of the increased osseous substance. Despite this fact, however, osteoclasts were as much as three times more numerous than usual and occasionally assumed giant proportions with bizarre stellate configuration.

The epiphyseal cartilage and primary trabeculae, therefore, differed morphologically in no essential manner from the normal. The fundamental variation appeared to be solely an increased number of secondary trabeculae. (Fig. 3.) Since these resulted from the deposition of osteoid by osteoblasts upon such framework as was available, it is reasonable to infer that the basic process in the development of the phosphorus line might simply be the persistence of an unusual number of primary trabeculae. Park, Jackson, Goodwin and Kadji[2] postulate that the deposition of lead in mineralizing cartilage impedes its resorption and therefore contributes to the production of the dense line in lead poisoning. It is our belief that elementary phosphorus affords similar interference with the resorbability of mineralized cartilage. This thesis was originally propounded by Kassowitz.[8] The presence of markedly increased numbers of osteoclasts implies an exaggerated attempt to overcome this induced resistance to absorption. Whatever the chemical background of the process, it is obvious that the persistence in excess of the primary trabeculae offered an overabundant framework for the deposition of bone. There was no evidence to support the contention that osteoblasts alone were stimulated to hyperfunction.[2] The appearance of the lesion suggested rather an increased activity of these cells in proportion only to the excess amount of superstructure available to them.

Roentgenograms exhibited evidence of increased radio-opacity in the subepiphyseal regions but there was lacking a sharpness of detail. It is possible that this resulted either from an inadequate dosage of phosphorus or from the fact that this element was administered up to the day of death and therefore insufficient time elapsed to permit separation of the phosphorus line from the epiphyseal cartilage. The sharply contrasting clear zone ordinarily present was thus obscured.

The changes observed consisted of a slight to marked increase in the density of the metaphyseal region increasing in width with the continued feeding of phosphorus. Minimal changes occasionally required comparison with films taken prior to the experimental period in order to establish conclusively the distinction of the line.

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[8] Kassowitz, M., *Z. f. Klin. Med.*, 1883, **7**, 93.

The degree of ossification was in these cases insufficient to produce obvious density. Histologic changes, however, were apparent in all animals receiving phosphorus for at least one week.

Summary and Conclusions. Twenty-two albino rats were given small amounts of elementary phosphorus supplementary to an adequate diet. Roentgenograms taken before and during the experimental period demonstrated the development of an area of increased density in the subepiphyseal region.

Histologic studies revealed a failure of resorption of the usual number of primary epiphyseal trabeculae. Since bone was deposited upon these persistent spicules in an otherwise normal fashion an apparent sclerosis was produced. Although the degree of ossification was distinctly greater than normal there was no evidence of direct stimulation of osteoblastic activity. The lesion was dependent entirely upon the unusual preservation of mineralized cartilage. It was thought that the persistence of these primary trabeculae was related to the influence of elementary phosphorus deposited in this region.

11744 P

Experimental Atherosclerosis and High Protein Diets.*

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The relationship of protein and experimental atherosclerosis has been summarized by Duff.[1] No clear cut experimental evidence was available that the feeding of proteins in conjunction with cholesterol promotes experimental atherosclerosis. Nor was there any indication that a sterol-free protein diet can produce atherosclerotic lesions.

In the course of a study on the possible effects of a high protein diet on cholesterol-induced arteriosclerosis, young adult chinchilla rabbits were fed one of 3 diets:

(A) A normal stock diet (15% digestible protein, dry weight) composed of white flour, alfalfa leaf meal, linseed oil meal, salt mixture, ground carrots, and water.

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* The earlier portion of this work has been aided by a grant from the Josiah Macy, Jr., Foundation.

[1] Duff, G. L., *Arch. Path.*, 1935, **20**, 81, 259.

(B) A high protein diet (approximately 38% protein) composed of essentially cholesterol-free defatted casein,† white flour, alfalfa leaf meal, salt mixture, ground carrots, and water.

(C) A high protein diet (approximately 39% protein) composed of white soy bean flour (Nusoy), linseed oil meal, alfalfa leaf meal, salt mixture, ground carrots, and water.

These diets were fed for 6 months (⅓ of the animals receiving the casein and soy bean diets were fed for 3 months only). Part of the rabbits on each diet received in addition a daily dose (60 or 250 mg) of cholesterol, dissolved in vegetable oil, mixed with the diet. Administration of cholesterol took place during the first 3 months of each period of feeding. Absorption was demonstrated by rise in blood cholesterol values. All animals gained weight and when sacrificed at the end of the experiment were sleek and well nourished. The aorta and viscera were studied histologically, using fat and elastic tissue stains on the rolled aorta and on the heart.

The experimental procedure and the results are summarized in Table I.

Three observations may be made from the above data. One is the occurrence of lesions indistinguishable from those of cholesterol sclerosis in rabbits fed a practically cholesterol-free (high protein-casein) diet.

The occurrence of atherosclerosis on an essentially cholesterol-free diet in two separate groups of rabbits used in consecutive years is of considerable interest, especially in view of the extreme rarity of this type of spontaneous lesion (Duff, *Arch. Path.*, 1935, **20**, 81).

TABLE I.
Summary of Feeding Experiments.

		Rabbits			Degree of sclerosis of aorta		
Diet	Chol. fed	No. used	No. sclerotic	% sclerotic	+	++	+++
Basic	None	8	0	0			
Casein	”	12	6	50	4	1	1
Soybean	”	8	0	0			
Basic	+	21	15	70	6	7	2
Casein	+	13	10	75		3	7
Soy bean	+	16	6	35	5	1	

+ = Lesion doubtful grossly, but present microscopically.
++ = One to several small atheromatous plaques visible grossly.
+++ = Numerous plaques present.

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† The casein was defatted by repeated extraction with hot alcohol according to the method of Sperry.[6] After extraction it sometimes still contained traces of cholesterol (0.024-0.107 mg in 100 g).

[6] Sperry, W. M., *J. B. C.*, 1926, **68**, 357.

The total amount of contaminating cholesterol ingested during 6 months in the casein-containing diet was approximately 2.7 mg. However, the plasma cholesterol of several of these rabbits became abnormally high, rising from less than 100 mg per 100 cc at the beginning of an experiment to 150 mg, and even 280 mg. Such a rise cannot be accounted for quantitatively by the small amount of cholesterol ingested. Since the rabbit normally does not ingest cholesterol, and does not absorb plant sterols,[2] the obvious conclusion is that synthesis of cholesterol was abnormally accelerated by the casein in the diet.

A second result is the increase in cholesterol sclerosis when cholesterol and casein are fed, compared to the incidence on a normal diet plus cholesterol. This increase is more apparent if the degree and extent of sclerosis are taken into consideration, rather than merely the number of animals affected. The differences shown in the table are rendered even more significant by the fact that over half of the rabbits fed the soy bean diet with cholesterol (10 of 16) and over half of those fed the basic diet with cholesterol (12 of 21) were given 250 mg of cholesterol daily, while all the animals on the casein diet with cholesterol received only 60 mg of the latter daily. Nevertheless the incidence and degree of sclerosis were highest in the last group.

The third significant observation is the apparent inhibition of lipid deposition in the aortas of rabbits fed soy bean and cholesterol. The mechanism of this is not clear. The thyroid glands of these rabbits did not differ grossly or histologically from those on the other diets. Furthermore, soy bean contains only traces of iodine.[3] The choline content of the soy bean diet is small (10 mg daily), and moreover is the same as that of the basic diet. Another lipotropic factor, methionin,[4] is present in greater amount in casein than in glycinin, the principal protein of soy beans.[5] There remains the possibility that some other factor is present in soy bean that restricts the deposition of cholesterol.

Summary. A diet high in soy bean flour diminishes the incidence and degree of experimental sclerosis in rabbits fed cholesterol, while a diet rich in defatted casein augments the degree of sclerosis. Rabbits, fed the high protein, defatted casein diet alone may develop atheromatous lesions indistinguishable from those produced by cholesterol.

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[2] Schönheimer, R., *Z. f. Physiol. Chem.*, 1929, **180**, 32.

[3] Adolph, W. H., and Ch'en, S. C., *Chinese J. Physiol.*, 1930, **4**, 437.

[4] Channon *et al.*, *Biochem. J.*, 1938, **32**, 977.

[5] Baernstein, H. D., *J. Biol. Chem.*, 1932, **97**, 663.

11745

Carrion's Disease II. Behavior of Bartonella Bacilliformis in the Developing Chick Embryo.

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Following the suggestion made by Goodpasture[1] numerous reports have appeared which have established the use of the developing chick embryo for the experimental study of numerous bacterial infections. We are reporting results obtained with the experimental infection of this host with *Bartonella bacilliformis* both in regard to the cultivation of this organism and to the course of the experimental infection so far as it has been observed.

Experimental. As a source of the infection 4 different strains of *Bartonella bacilliformis* were used. They were maintained in Noguchi's leptospira medium, cystine blood agar and in glycerine blood agar.

In the course of the experimental work it was found that blood-glucose-cystine agar as devised by Francis for the cultivation of *B. tularense* provided an excellent medium for the rapid growth of this organism. The only modification in the preparation of this medium which was introduced was to omit heating the flask of prepared medium for 2 hr. at 60°C.

Ordinary blood agar to which 1% glycerine was added also proved to be a very favorable medium for growth. In this medium rapid proliferation occurs within 48 to 72 hours at room temperature, 25-28°C. The organism grows as a thin, almost imperceptible film over the surface of the agar. Smears stained by Giemsa's method reveal the organisms in great abundance. These media have been found to be much more satisfactory to handle and provide for a much more rapid and luxurious growth than does Noguchi's leptospira medium.

Chick embryos of 8- and 12-day incubation were used. The chorio-allantois, the allantoic fluid, the amniotic fluid and the yolk sac were investigated as to their relative suitableness for the proliferation of this organism. The embryos were inoculated by the cover-slip technic as described by Goodpasture and Buddingh.[2] Incubation was carried out at 37°C and at room temperature, 25-28°C.

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[1] Goodpasture, E. W., *Southern M. J.*, 1933, **26**, 418.

[2] Goodpasture, E. W., and Buddingh, G. John, *Am. J. Hyg.*, 1935, **21**, 319.

The course of the infection was followed by examining smears stained by Giemsa's method made from the inoculated areas at daily intervals and was further controlled by inoculation of cystine blood agar, or blood glycerine agar as well as in plain infusion broth.

The lesions developing in the chorio-allantois were fixed in Zenker's-acetic acid fluid, embedded in paraffin and sections were stained with hematoxylin and eosin and also by Giemsa's method.

It was found that room temperature (25-28°C) was more favorable for the rapid proliferation of the organism than 37°C. The lower temperature has the disadvantage in that there is a higher mortality of the embryos and secondary contamination occurs more frequently.

The best growth of the organism was obtained in the chorio-allantoic fluid. This was readily inoculated by inserting a needle through the chorio-allantois and introducing the inoculum by means of a syringe. In this medium the organism is found to proliferate with great rapidity. Twenty-four hours after inoculation stained smears made by withdrawing a drop of the fluid with a capillary pipette reveal numerous bacillary forms diffusely dispersed but showing a marked tendency toward clumping which appears to begin about a nidus of cellular debris or fibrin strands. At 72 hours the allantoic fluid is grossly turbid. Great numbers of organisms are present in smears. They appear as small bacillary forms, usually closely clumped together in large masses. A few polymorphonuclears, monocytes and red blood cells are also present. The organisms have been found to continue proliferation as long as 6 days following inoculation. Transfers to the chorio-allantoic fluid were usually made at 72 hours. In this manner the organism has been maintained through 6 successive passages in this medium.

It was found that chorio-allantoic fluid aspirated from the embryo by means of a sterile capillary pipette and transferred to test tubes also provided an excellent medium for cultivating this organism. Growth is not quite as rapid as *in vivo,* but the *in vitro* cultures show large numbers of organisms at 48 hours and beyond. Transfers to fresh tubes of chorio-allantoic fluid can be successfully made.

Growth of the micro-organism is also obtained following inoculation of the yolk sac, as introduced by Cox[3] for the rickettsiae. Smears show the organisms in abundance in 48 to 72 hours. A few organisms can occasionally be found within the cells of the yolk membrane, but the preponderance of the growth takes place within the yolk fluids. Thus far inoculation of the amniotic fluid has not met with any considerable success.

The chorio-allantois was infected by simply dropping a small amount of the suspended organisms onto the surface of the membrane and also in many instances by producing slight injury to the membrane by light scarification with the point of the needle at the time of inoculation. The course of the infection was followed by preparing stained smears of scrapings from the surface of the membrane.

During the first 48 hours following introduction of the inoculum without scarification there is a marked inflammatory response which at first is composed chiefly of polymorphonuclear leukocytes but later is predominantly made up of large mononuclears. In the earlier stages the micro-organisms are present as free clumps but later they are mostly found within the cytoplasm of the mononuclear cells. This cytologic picture persists in smears until the 5th or 6th day, after which the parasite is no longer demonstrable. Grossly, only slight thickening by edema and clouding of the inoculated area are observed during the first 4 days. These changes then rapidly disappear.

When slight mechanical injury to the membrane is added at the time of inoculation the infection presents a different appearance. There is a rapidly developing edema during the first 24 hours. Numerous small round depressions appear on the surface of the inoculated area. These have a thickened edematous border. At 48 hours the rim of these shallow craters develops numerous small pinpoint to pinhead sized granulations, many of which have vesicular centers. These gradually develop into firm white opaque nodules which attain their maximum intensity between 72 and 96 hours. These granular proliferations are also often observed in the bottom of the depressions. After the 4th day they gradually disappear so that by the 7th day only a thin linear depression is present on the surface of the membrane. The granular lesion is also often found dispersed over the surface of the membrane in areas where no crater-like depressions are present.

Smears from scrapings of these lesions show the same cytological picture as described above. The number of micro-organisms present, however, is much greater.

Attempts to transfer the infection in series by inoculation of the ground lesions were successful only for one passage. Further passages than the second failed to produce any signs of infection. Inoculations of emulsion of the lesions of the 2nd passage on suitable artificial medium were positive up to the 4th day following inoculation.

In embryos inoculated on the chorio-allantois in which no organisms could be demonstrated in scrapings from the membrane the organism could always be demonstrated in smears from the allantoic fluid and also by culture of the fluid on suitable artificial medium.

A limited number of membranal lesions were examnied histologically. The reaction is limited chiefly to the mesodermal layer. There is considerable edema, and numerous foci of hemorrhage are scattered throughout. The ectodermal epithelium is generally well preserved except at such points where there has been mechanical injury. In these areas there is active regeneration of the epithelium at the edges of the destroyed ectoderm.

The lesion in the mesoderm is predominantly perivascular consisting of proliferated fibroblasts, mononuclears and an occasional polymorphonuclear. In areas of such foci, adjacent to the ectoderm, there is necrosis consisting of hyalinized cellular debris, and numerous leukocytes. Surrounding these necrotic foci is a prominent border of large mononuclear cells and numerous multinucleated giant cells of the foreign body type. Within the mononuclears in these foci typical Bartonella are found. They are occasionally found within the cytoplasm of the giant cells. These focal cellular proliferations correspond to the granular lesions observed in the gross. These granulomatous lesions in the mesoderm are richly supplied with newly formed capillaries.

Although all the essential characteristics of the human Carrionic cutaneous granuloma are not present in this experimental

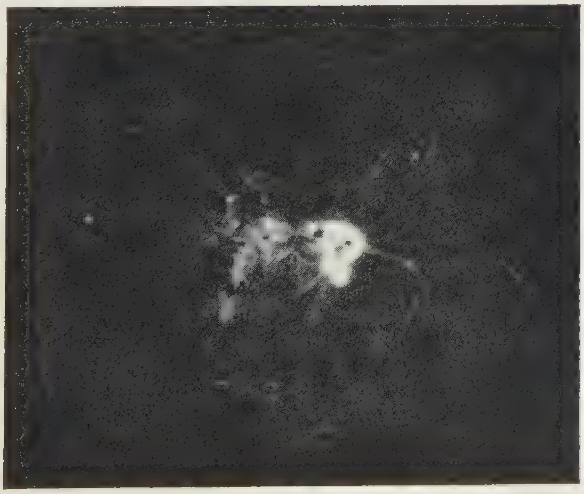

FIG. 1.

Gross appearance of 4-day lesion in the chorio-allantois produced by *B. bacilliformis.*

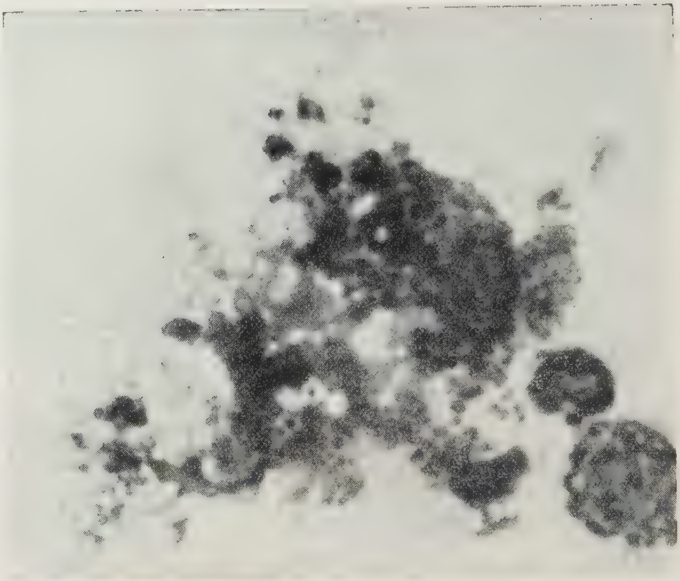

FIG. 2.

Giemsa stained smear preparation from 4-day lesion in the chorio-allantois. × 1800.

lesion its formation is very similar especially in regard to its granulomatous angioblastic features and also in respect to the presence of the etiological agent.

The presence of giant cells is rarely observed in the human lesion and these are interpreted as vascular buds. In the membrane they probably represent a foreign body reaction in response to the presence of the inoculum.

Discussion. Pinkerton and Weinman[4] are of the opinion that *B. bacilliformis* is a facultative intracellular parasite which will proliferate extra and intracellularly in media containing growing or surviving cells *in vitro* in contradistinction to the rickettsiae which are more strictly intracellular parasites. Cox,[3] on the other hand, has stressed the extracellular growth of rickettsiae which he has successfully cultivated in the yolk sac of the developing hen's egg. An analogous circumstance has been observed with *B. bacilliformis* in relation to its growth in the fluid and the membrane of the yolk sac.

Summary. The observations reported here indicate that *Bartonella bacilliformis* is readily cultivated within the allantoic fluid of the developing chick maintained at room temperature, 25-28°C.

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[3] Cox, R. H., *Public Health Rep.*, 1938, **53**, 2241.

[4] Pinkerton, H., and Weinman, D., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 587.

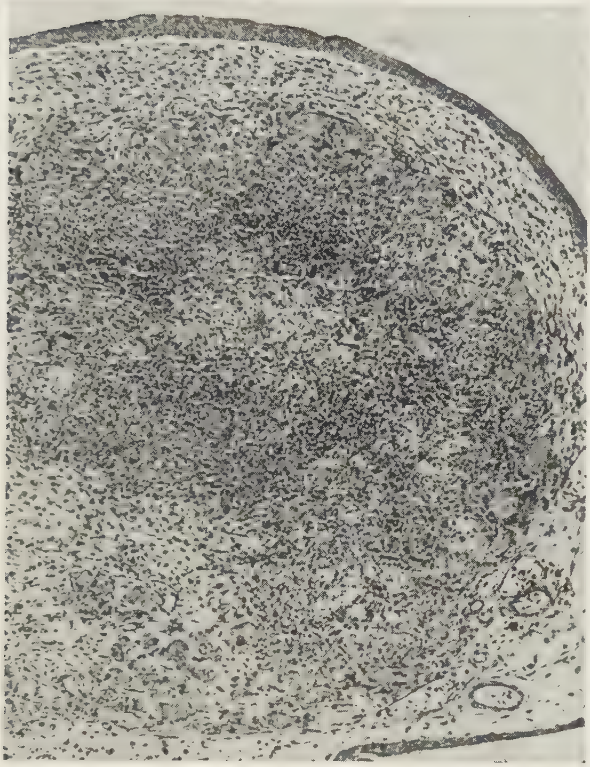

FIG. 3.
Microscopic appearance of 4-day lesion in the chorio-allantois. Hematoxylin and eosin. × 120.

The growth of *B. bacilliformis* in this environment is much more rapid and abundant than in any of the suitable artificial media. Furthermore, the allantoic fluid of the embryo is a suitable medium for the growth *in vitro* of this micro-organism.

The pleomorphism of this organism is again confirmed and the observations here reported are in accord with the interpretation of Pinkerton and Weinman[4] who regard the various morphological aspects of the organism as expressions of pleomorphism rather than as representing stages in a progressive life cycle.

The chorio-allantois thus far has proven to be unsuitable for the transfer in series of *B. bacilliformis.* However, the first passage direct from artificial medium with this pathogen induces the development of a granulomatous lesion the histological features of which bear a close resemblance to that of the human cutaneous infection (verruca) and in which the presence of the etiological agent can be demonstrated.

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Influence of pH on Absorption of Nicotine from Urinary Bladder and Subcutaneous Tissues.

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It has been shown that the pH plays an important rôle in the absorption of alkaloids from the stomach.[1] So far as we know, no studies are available as to the influence of this factor on the absorption of these compounds from the urinary bladder. It seemed likely, however, that this membrane might behave like the gastro-intestinal mucosa and absorb alkaloids quite rapidly from alkaline solutions. Nicotine was selected for the study because of the possibility that its reabsorption from the urinary bladder may be a source of poisoning in heavy tobacco smokers when the urine becomes alkaline.[2]

Experiments were carried out, therefore, to determine the influence of the pH on the absorption of nicotine from the urinary bladder. Under light ether anesthesia the ureters were ligated in 10 cats and a cannula inserted in the urethra for injection. The bladder was washed with physiological salt solution, and nicotine was injected in a buffered solution. Phthalates, phosphates and sodium bicarbonate were used as buffers. The volume of fluid injected in each experiment was 5 cc per kg. The pH of the solution injected into the bladder and of that recovered from the bladder 4 hours later or at death was determined electrometrically (glass electrode).

The results are shown in Table I. Figures are given for the time of onset and duration of the advancement of the nictitating membrane; the duration of this effect parallels closely the degree of nicotine poisoning. It may be seen that at pH levels of about 4.5 to 6.0, doses of 10 and 20 mg of nicotine produced no effects. Similar doses at pH levels of about 7.0 to 7.9 produced fairly marked poisoning; advancement of the nictitating membrane appeared in 20 to 30 minutes after injection and was present for an average of 53 minutes. At pH levels of about 8.0 to 9.0, 10 mg caused very severe

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* With the assistance of Charles Salzman, B.S.

[1] Travell, J., *J. Pharm. and Exp. Ther.*, 1940, **69**, 21.

[2] Travell, J., Bodansky, O., and Gold, H., *J. Pharm. and Exp. Ther., Proc.*, 1940, **69**, 307.

TABLE I.
Influence of pH on Absorption of Nicotine from Ligated Urinary Bladder.

Cat No.	pH of solution		Dose of nicotine injected into bladder[†] mg per kg	Advancement of nictitating membrane		Degree of nicotine poisoning	Calculated concentration of undissociated nicotine base in solution injected
	injected into bladder	recovered from bladder[*]		onset, min	duration, min		
1	4.6	4.7	10	—	0	None	1:1,250,000
2	5.4	5.8	20	—	0	”	1:83,000
3	7.2	7.0	10	24	19	Moderate	1:3,300
4	7.9	7.4	10	21	50	Marked	1:1,000
5	7.0	6.8	20	30±	54	”	1:2,500
6	7.9	7.0	20	20	90	”	1:520
7	8.6	8.1	10	5	180	Very marked	1:630
8	8.7	8.1	10	10	148	” ”	1:590
9	9.0	9.0	20	5	—	Fatal 17 min	1:270
10	9.0	8.9	20	6	—	” 40 ”	1:270

[*] 4 hours after injection or at death.
[†] Injected in a volume of 5 cc per kg.

nicotine poisoning; advancement of the nictitating membrane appeared much sooner and persisted for a much longer period of time, 2½ to 3 hours. At these latter pH levels 20 mg proved fatal. These results indicate that nicotine may be absorbed by the urinary bladder and that within a certain range the higher the pH, the more rapid is its absorption.

In Table I a correlation is shown between the degree of nicotine poisoning and the concentration of undissociated nicotine base. The latter was calculated in accordance with the formula which we have previously derived,[1] taking the dissociation constant of nicotine[3] as 9 x 70^{-7}.[†] At the pH values used in these experiments, the percent of total alkaloid present as undissociated base varied through a wide range: approximately 0.04% at pH 4.5, 0.2% at pH 5.0, 1% at pH 6.0, 10% at pH 7.0, 52% at pH 8.0, and 92% at pH 9.0. Taking into account the dissociation and the dose of nicotine and the volume of the solution, the concentration of undissociated nicotine base in the solution injected into the bladder was found to range from 1:1,250,000 in Cat 1 to 1:270 in Cats 9 and 10. At the lowest concentrations of undissociated base, 1:83,000 or lower, no nicotine effects were observed. At concentrations of about 1:3,000 to 1:1,000, moderate to marked poisoning occurred; the average duration of advancement of the nictitating membrane was 41 minutes. At a concentration of about 1:600, nicotine effects were more

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[3] Craig, L. C., and Hixon, R. M., *J. Am. Chem. Soc.*, 1931, **53**, 4367.

[†] Thus, $$\frac{[\text{Undissociated nicotine}]}{[\text{Nicotine ions}]} = \frac{1 \times 10^{-14}}{(9 \times 10^{-7})\ (1 \times 10^{-\text{pH}})}$$

marked and the average duration of the nictitating membrane advancement was 139 minutes. At a concentration of 1:270, death occurred. These results indicate that the rate of absorption of nicotine from the urinary bladder depends to a large extent on the concentration of undissociated nicotine base.

Two experiments were reported[1] in which nicotine was injected into the ligated stomach of the cat. Twenty-four hours after the injection of 50 mg per kg in acid solution (pH 1.2), the pH of the gastric fluid was 4.2, and nicotine effects had not been observed. Injected at a pH of 8.6, 20 mg per kg was fatal in 41 minutes. In these experiments the volume of fluid was 15 cc per kg, and the calculated concentration of undissociated nicotine base in the solution injected was approximately 1:15,000,000 in the case of the acid medium, and 1:900 in the case of the alkaline medium. As we have seen, concentrations of the base of about 1:500 to 1:1,000 injected into the urinary bladder caused severe poisoning, but did not prove fatal. However, if allowance is made for the greater absorbing surface of the stomach as compared with that of the bladder, the rates of absorption of nicotine by the membranes of these 2 organs appear to be of the same order.

The influence of the hydrogen ion concentration on the speed of absorption of an alkaloid from the subcutaneous tissues has not heretofore been studied directly, but Moore and Row[4] found nicotine base to be 5 times as active as its hydrochloride when injected subcutaneously in the frog. Haag and Neale[5] more recently reported that the toxicity of nicotine[‡] injected subcutaneously in mice was only slightly greater than that of nicotine sulfate or salicylate. We have found that when nicotine is injected subcutaneously (within the inguinal fold of skin) in cats in a buffered solution in a constant volume of fluid, 5 cc per kg as employed in the bladder, the pH plays a rôle in the speed of absorption as indicated by the interval to death, and by the M.L.D. (Table II). When the pH was reduced from an alkaline to an acid value and at the same time the dose of nicotine was increased by 33%, the average interval to death was prolonged from 32 minutes to 102 minutes. When the pH was similarly reduced from 9.0 to 4.8 but the dose of nicotine increased by 66%, the average interval to death was the same, namely, 32 minutes at the acid and alkaline pH. The dose of nicotine which proved fatal in the majority of animals when injected at an alkaline

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[4] Moore, B., and Row, R., *J. Physiol.*, 1897, **22**, 273.

[5] Haag, H. B., and Neale, R. C., *J. Pharm. and Exp. Ther., Proc.*, 1940, **69**, 289.

[‡] We have determined that a 1% solution of nicotine in the form of the base has a pH of about 10.1, and in the form of the sulfate, a pH of about 4.3.

TABLE II.
Influence of pH on Absorption of Nicotine from Subcutaneous Tissues.

Dose of nicotine* mg per kg	pH of solution injected	No. cats	No. died	Avg time injection to death, min
6	9.0	6	0	—
7.5	9.0	6	0	—
9	9.0	6	5	32 (25-36)
9	4.8	6	0	—
12	4.8	6	2	102 (50-304)
12	6.4	6	3	
15	4.8	6	4	32 (24-50)

*Injected in a volume of 5 cc per kg.

pH was 9 mg per kg, and at an acid pH was 15 mg per kg; 9 mg per kg injected at an acid pH was fatal in none. Thus within a certain critical range of pH and dosage both the M.L.D. of nicotine and the interval to death may be materially increased by lowering the pH of the solution injected. The use of a buffer in our experiments might explain the apparent discrepancy between these results and those of Haag and Neale.[5]

These experiments indicate that the absorption of nicotine is greater from subcutaneous tissues than from the urinary bladder, when injected at similar pH values and in the same volume of fluid. In the case of the urinary bladder, the amount of fluid injected remained unchanged, and hence as nicotine was absorbed, its concentration necessarily decreased. This fact would result in a diminishing rate of absorption, and provided the initial concentration was not high enough to cause death, nicotine effects would disappear while appreciable amounts of nicotine still remained in the bladder. When given by the subcutaneous route, the fluid was rapidly absorbed; this fact would tend to maintain the original concentration of nicotine, to prevent the slowing down of absorption and to insure virtually complete absorption of the alkaloid. That in the latter case there may actually be a rising curve of absorption is shown by the fact that in one experiment 20 mg per kg injected subcutaneously at a pH of 7.0 caused death after as long as 5 hours. In this case the increasing rate of absorption could scarcely be explained by a changing pH. It is obvious that owing to the number of variable factors a comparison cannot be made as to the influence of the pH alone on the speed of absorption of nicotine from subcutaneous tissues and that from the urinary bladder.

Summary. These experiments show that the hydrogen ion concentration plays an important rôle in the absorption of nicotine from both the urinary bladder and the subcutaneous tissues. The toxicity of nicotine given by these routes is appreciably decreased when the pH of the solution is reduced within a certain range. At similar pH levels nicotine is absorbed from the urinary bladder and from the stomach at essentially similar rates. Absorption of nicotine from the subcutaneous tissues is greater than from the urinary bladder when the alkaloid is injected at similar pH values, but the impossibility of controlling the factors which influence absorption from the subcutaneous tissues precludes direct comparison as to the rôle of the pH alone.

A correlation is shown between the rate of absorption of nicotine from the urinary bladder and the calculated concentration of undissociated nicotine base in the solution injected.

The ligated urinary bladder provides a technic for the study of factors influencing absorption which is in some respects more satisfactory than the ligated stomach or intestinal loop, owing to the greater ease of controlling in the bladder certain variable factors such as the volume of fluid and the pH.

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Effect of N′-Dodecanoylsulfanilamide and of Sulfapyridine on Experimental Tuberculosis in Rabbits.*

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N′dodecanoylsulfanilamide was synthesized by Crossley, Northey, and Hultquist[1] and proposed as a possible chemotherapeutic agent against tuberculous infections. Climenko[2] has recently shown it to be capable of inhibiting the growth of human tubercle bacilli *in vitro*. He also reported that it exerts an inhibitory effect against the development of experimental tuberculosis in guinea pigs infected with a human strain of tubercle bacilli. Steinbach and Duca,[3] however, failed to demonstrate an inhibitory effect by this drug in experimental tuberculosis in guinea pigs. In both of the investigations the H-37 strain of human tubercle bacilli was used and daily doses of 100 mg N-dodecanoylsulfanilamide in 2% olive oil solution were administered to the animals for from 30 to 45 days. Sulfapyridine in olive oil was also administered in the same dosage by Steinbach and Duca and this drug was reported by them to be equally ineffective.

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* This study was aided by a gift from the late Mr. George L. Bourne. The dodecanoylsulfanilamide and the sulfapyridine used in this experiment were supplied by the Calco Chemical Company through the courtesy of Dr. D. A. Bryce.

[1] Crossley, M. L., Northey, E. H., and Hultquist, M. E., *J. Am. Chem. Soc.*, 1939, **61**, 2950.

[2] Climenko, D. R., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 624.

The present communication records the results of the administration of N-dodecanoylsulfanilamide and sulfapyridine in olive oil suspension to rabbits infected with the Ravenel strain of bovine tubercle bacilli. The infecting dose of bacilli was 0.001 mg inoculated intravenously. A daily dose of 1.6 g of either drug was administered in divided doses of 400 mg suspended in 2 cc of olive oil and fed by means of a dropper. Sixty male albino rabbits weighing from 2 to 3 kg each were infected and divided into groups for treatment as follows:

Group A. 24 animals, given 400 mg N-dodecanoylsulfanilamide 4 times daily for 57 days.

Treatment started 1 day after infection—6 animals
” ” 10 ” ” ” 6 ”
” ” 20 ” ” ” 6 ”
” ” 35 ” ” ” 6 ”

Group B. 24 animals, given 400 mg sulfapyridine 4 times daily for 57 days.

Treatment started 1 day after infection—6 animals
” ” 10 ” ” ” 6 ”
” ” 20 ” ” ” 6 ”
” ” 35 ” ” ” 6 ”

Group C. 12 animals, untreated controls.

One animal of the control group was discarded on the first day after infection because of severe diarrhea. A second animal of Group C was sacrificed 64 days after infection. With the exception of one animal in Group A which lived until 326 days after infection when it was sacrificed the remainder died within a period of from 14 to 272 days after infection. The control animal sacrificed at 64 days revealed no gross or microscopic evidence of tuberculosis and all of the animals which died within 65 days revealed no evidence of tuberculosis or, at most, a few isolated small tubercles. There were 9 animals which died within this period. The cause of death in some of those may have been the toxic effect of the drug as paralytic manifestations were frequently the precursors of death. All of the animals which lived for more than 65 days showed gross evidence of tuberculosis except the one animal in Group A which survived for 326 days when it was sacrificed and revealed no gross or microscopic evidence of tuberculosis. The explanation of this exception is not clear, and because of its marked disparity with the general results it probably should be regarded as not significant. The majority of both the treated and the control animals that lived for more than 65 days after infection had generalized tuberculosis of sufficient extent to be the cause of death. In many there was massive caseation of the lungs, the organ which was usually the most extensively involved. Table I shows the probable causes of death of the animals in the 3 groups which survived for longer than 65 days after infection. The percentage of those in each group which apparently died of tuberculosis is shown in the table with the average survival period. It is apparent that there is little difference between the treated groups and the controls. The somewhat lower percentage of deaths caused by tuberculosis in the controls and the somewhat longer average survival period may be due to the absence of toxic effects of the drugs which were given in very large doses to the treated groups. The relatively small number of controls, however, makes the possible significance of the difference uncertain.

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[3] Steinbach, M. M., and Duca, C. J., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 133.

The extent of tuberculous involvement of the separate organs and of the total disease in each animal was graded on a scale varying from + to ++++. This served as a basis for the determination of the probable causes of death. The grading also gave a means of comparison between the sub-groups of 6 animals in Groups A and B, whose treatments were started at varying intervals after infection. In this comparison there appeared to be no significant differences attributable to the time when treatment was begun.

TABLE I.

		Probable causes of death						
				Tuberculosis. Massive tbc of lungs or extensive generalized tbc sufficient to cause death				
	No. of animals	Sacrificed. No evidence of tbc	Non-tbc causes. Extent of tbc insufficient to cause death	Massive	Extensive	Total	%	Avg duration of life
Group A	20	1 (326th day)	4	12	3	15	75.0	188 days
Group B	21	0	6	10	5	15	71.4	176 ”
Group C	8	0	3	5	0	5	62.5	213 ”

Analysis of causes of death of animals which lived longer than 65 days following intravenous inoculation with bovine tubercle bacilli. Group A—treated with N′-dodecanoylsulfanilamide; Group B—treated with sulfapyridine; Group C—untreated controls.

The concentration of drug in the blood was determined by Marshall's method[4] in a number of animals of Groups A and B. The values varied from 1.2 mg % to 3.6 mg % of the free drug calculated as sulfanilamide. Those animals which exhibited higher blood levels appeared to develop as extensive disease as those which exhibited low levels. Calculi were found in the kidney pelves of several of the animals in Group B and in the ureters of one of these animals. No renal calculi were found in any of the animals treated with N′-dodecanoylsulfanilamide, or in the controls.

Conclusions. N′-Dodecanoylsulfanilamide and sulfapyridine administered in large doses failed to exert any demonstrable inhibitory effect on experimental tuberculosis in rabbits infected with a bovine strain of tubercle bacilli.

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Electrophotometric Determination of Phosphorus by the Method of Fiske and Subbarow.

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The substitution of the filter photometer for the visual matching of solutions directs attention forcibly to the fact that many of the reactions employed in quantitative colorimetric microanalysis yield colors that are highly variable with respect to their rates of intensification and fading. An attempt to carry out Bodansky's directions[1] for the determination of serum phosphatase activity with a filter photometer* demonstrated the procedure of Kuttner and Lichtenstein[3] for the determination of phosphorus to be particularly troublesome in this respect. The essay was made during an excessively hot and humid period of the summer when the daily fluctuations of temperature sometimes amounted to 10°C. Under these circumstances it was found impossible to reproduce with satisfactory accuracy from day to day the standard curves expressing the relation of the phosphorus concentration of the solutions to their absorption of light. The difficulty manifested itself in the irregularity of the values that were obtained for K_1, the proportionality constant in the familiar equation, $C = 2—\log G/K_1$, where C is the concentration of phosphorus; 2, the logarithm of 100, representing complete transmission of light; and G, the reading of the galvanometer. Efforts to modify the influence of temperature by icing the solutions before mixing were defeated by the condensation of a film of moisture on the absorption tubes.

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[4] Marshall, E. K., Jr., *J. Biol. Chem.*, 1937, **122**, 263.

[1] Bodansky, A., *J. Biol. Chem.*, 1932, **99**, 197; 1933, **101**, 93; 1937, **120**, 167.

* The instrument was constructed in this laboratory. It employs a Lange selenium photocell, type S 50.[2] The absorption tubes are matched test tubes, 20 x 175 mm. The source of light is an automobile headlight bulb; a current of approximately 1.6 amperes is drawn from a storage battery that is under a continuous trickle charge. A Lange "Multiflex" galvanometer is used.

[2] Lange, B., translated by St. John, A., *Photoelements and Their Application*, New York, Reinhold Publishing Corporation, 1938.

[3] Kuttner, T., and Lichtenstein, L., *J. Biol. Chem.*, 1932, **95**, 661.

The replacement of stannous chloride, the reducing agent in the Kuttner and Lichtenstein method, by 1-amino-2-naphthol-4-sulfonic acid, as recommended by Fiske and Subbarow,[4] led to more reproducible results; the effect of fluctuations of temperature was comparatively inconspicuous. However, the blue color did not attain maximum intensity within a short time, as we had hoped, but gained in intensity at a diminishing rate for as long as 72 hours, the limit to which we continued our observations. Hence, in order to attain the required degree of consistency it was necessary to make the measurements of absorption of light within a rather limited time interval selected arbitrarily with reference to the moment of mixing the solutions.

Considerable independence of timed readings and demonstrable increase of reproducibility was attained by recourse to a principle the application of which was suggested by Sunderman and Razek.[5] These investigators called attention to the fact that intensification or fading of a colored solution does not necessarily signify that the absorption of light in each and every region of the transmission spectrum of the solution is undergoing alteration. They demonstrated that with the passage of time the blue color that is developed in Benedict's sugar method[6] absorbs progressively more light of wave lengths between 400 and 580 millimicrons and progressively less in the region between 600 and 700 millimicrons; in a narrow zone bordering on 590 millimicrons there is no appreciable change with time, the degree of absorption in this region remaining constantly proportional to the concentration of sugar present in the solution.

Accordingly, we obtained serial curves representing the transmission of visible light by the Fiske and Subbarow blue color. The curves were traced at 15, 30, 75, 120, and 170 minutes after the solutions were prepared. The Hardy recording spectrophotometer was employed; each tracing required about 2 minutes to complete.† Comparison of these curves revealed (Fig. 1) that whereas a considerable increase of absorption of light occurred in the red region of the spectrum, the amount of absorption in a zone between 400 and 420 millimicrons in the violet portion of the spectrum remained appreciably more constant. On the basis of this information a combination of filters was selected that isolated a band extending in the visible spectrum from 400 to 430 millimicrons. The elements of the combination were obtained from the Corning Glass Company. The individual components were: Light Shade Aklo, No. 396,‡ thickness, 2.0 mm; Violet, No. 511, thickness, 3.0 mm; Red Purple Ultra, No. 597, thickness, 3.3 mm. The extended transmission

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[4] Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, **66**, 375.

[5] Sunderman, F. W., and Razek, J., *J. Biol. Chem.*, 1937, **118**, 397.

[6] Benedict, S. R., *J. Biol. Chem.*, 1931, **92**, 141.

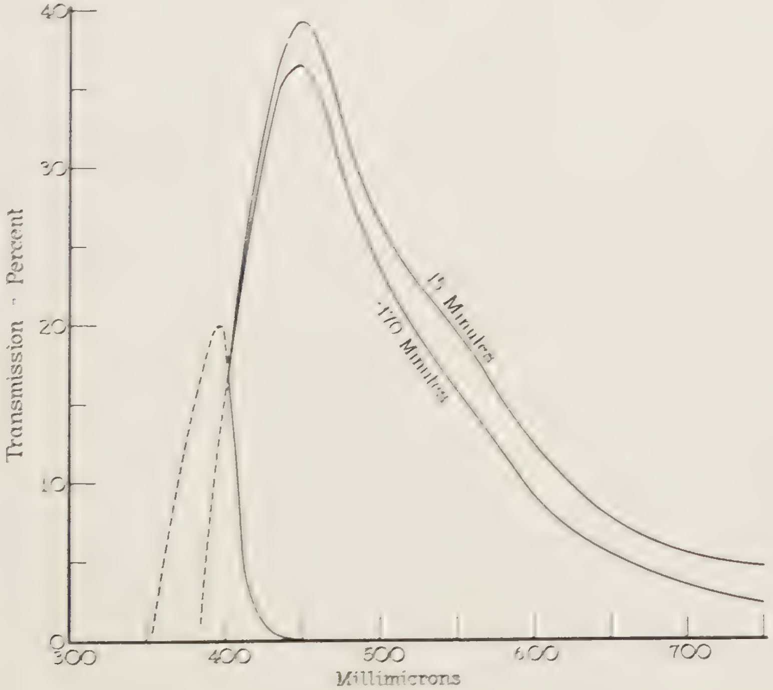

FIG. 1.

Per cent transmission in the visible spectrum (solid lines) and in the ultraviolet region (broken lines) by the Fiske and Subbarow blue color at 15 and 170 minutes after mixing the solutions, and by the filter pack.

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† The Electrical Testing Laboratories, East End Avenue and 79th St., New York City.

‡ The numbers are the manufacturer's designation.

curves of the solution and the filter pack§ demonstrated that both media transmitted light in the ultraviolet region (Fig. 1). The extent to which transmission in this region changed with time was not investigated.

With these filters in place the solutions showed a slight but readily appreciable increase of intensity during 30 minutes after mixing but the change during the ensuing 60 minutes was negligible. Accordingly we have made it a practice to carry out the measurements of light absorption at any time within an hour, commencing 30 minutes after the solutions are prepared; this period may be extended to 3 hours without substantial loss of accuracy.

The absorption of light in the region isolated has been found to be such that even extremely pale solutions produce satisfactory deflection of the galvanometer. At the same time it is not so great that the concentration of phosphorus must be kept extremely low in order to avoid galvanometer readings that fall below the 10% mark on the scale. It requires about 700 μg per 100 cc of solution as introduced into the absorption tubes to yield this value. When plotted on semilogarithmic coördinates the regression line of galvanometer reading on concentration is so nearly linear throughout its entire extent that the regions between the 5 points that are determined in its preparation can be considered to be strictly linear without introducing significant error.

With these provisions, we have found it possible to make rapid and precise measurements of inorganic phosphorus by the method of Fiske and Subbarow with 0.2 cc of serum and of phosphatase activity by Bodansky's method with an additional 0.2 cc. Duplicate determinations require twice as much.

The precision of the technic is illustrated by the fact that the standard error of measurement (S.E.$_{M}$) of the mean of 232 determinations of serum inorganic phosphorus in duplicate has been calculated to be 0.05 mg per 100 cc. This value was found to be consistent throughout a range that extended from 1 to 13 mg per 100 cc, indicating in approximate terms a 5% error at lowest concentrations, a 1% error at modal concentrations and an error of 0.5% at the higher concentrations. The S.E.$_{M}$ of the mean of 235 duplicate determinations of phosphatase varied from 0.2 Bodansky units in the range from 5 to 10 units to 0.6 units in the range from 40 to 90 units; thus the percent error varied between 3.2 and 0.9 as the amount of measured phosphatase activity increased.

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§ Dr. Hugh Darby of the Department of Biochemistry kindly measured the transmission with a hydrogen discharge tube and a Spekker photometer in conjunction with a Hilger medium spectrograph.

11749

Complement Titer of Blood of the Newborn.

P. Wasserman and E. Alberts. (Introduced by I. A. Mirsky.)
From the May Institute for Medical Research of the Jewish Hospital, Cincinnati, O.

During the course of studies on the complement content of human blood, we noted that the cord blood of the newborn infants consistently was of a lower titer than that of adults. We, therefore, determined simultaneously the complement titer of a series of newborn (cord) bloods and of the mother's blood taken at the time of delivery.

The titration was made according to the method outlined by Ecker, *et al.*[1] The serum was diluted 1 to 15 and a series of tubes set up starting with 0.01 cc of the diluted serum and increasing by

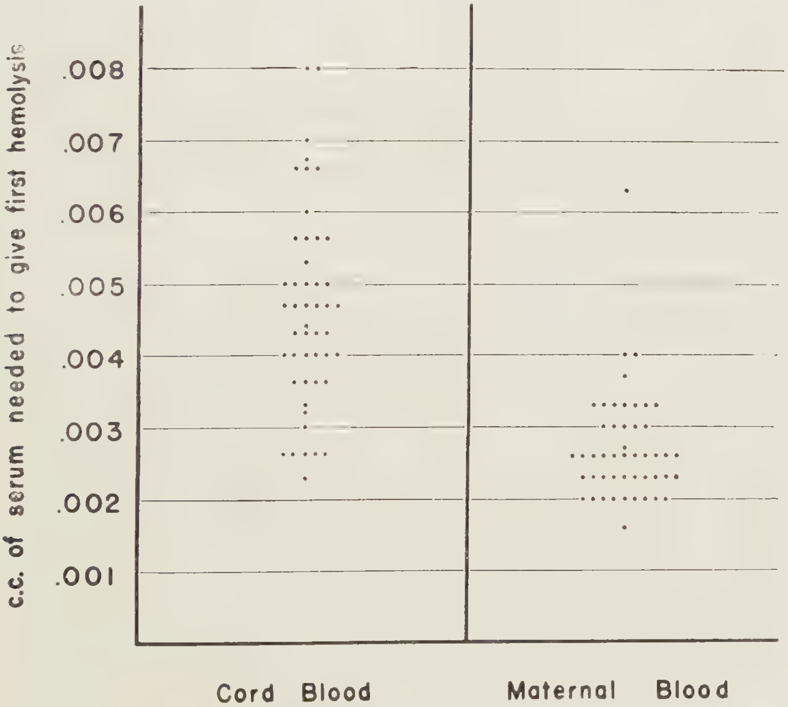

FIG. 1.
The comparative complement content in human newborn (cord) blood and maternal blood.

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[1] Ecker, E. E., Pillemer, L., Wertheimer, D., and Gradis, H., *J. Immunology*, 1938, **34**, 19.

0.01 cc to reach 0.15 cc. The volume in all tubes was adjusted to 0.15 cc with saline and 1 cc of cells sensitized with 5 units of hemolysin was added. The tubes were incubated at 37°C for 30 minutes, centrifuged and examined for the point of first hemolysis which was taken as the end point. The volume of the undiluted serum necessary for this first hemolysis was considered as the index of the complement titer.

The comparative titers of the 48 samples are illustrated in Fig. 1 The cord blood titer varies considerably, ranging from 0.0023 to 0.0080, with a mean of 0.0046. The maternal bloods, which had in each case been drawn immediately after delivery, presented titers ranging from 0.0016 to 0.004 with but one exception which fell to 0.0063. The mean for the maternal blood was 0.0030.

Statistical analysis of the data reveals that a "reliability of difference" of 7 exists between the complement titer of maternal and newborn (cord) blood. This definitely indicates that a distinctly smaller amount of complement is present in the newborn than in the adult.

Of perhaps greater significance than the means is the fact that a comparison of the cord and maternal bloods of individual instances almost invariably reveals a higher complement titer in the maternal blood. In several instances, both bloods contained the same amount of complement, but in no instance did the cord blood show a higher value than that of the maternal blood.

This observation is essentially similar to that reached by Solling[2] and by Nattan-Larrier, *et al.*,[3] who used, as a measure of the titer, the volume of serum necessary for complete hemolysis. Further work, using both animal and human sera, is in progress in an attempt to determine at what age and under which circumstances the serum in the newborn rises to the adult level, and also to determine the significance of the low complement titer of the newborn.

We wish to acknowledge the coöperation of Dr. H. L. Woodward, who permitted the use of his service at the Cincinnati General Hospital as a source of material.

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[2] Solling, P., *Z. f. Immunitatsf. u. exp. Ther.*, 1937, **91**, 15.

[3] Nattan-Larrier, L., Grimard, L., and Dufour, J., *Compt. Rend. Soc. de Biol.*, 1937, **125**, 358.

11750

Pantothenic and Hydroxypantothenic Acids in Animal Nutrition.

Elisabeth Zschiesche and Herschel K. Mitchell. (Introduced by R. R. Williams.)

From Private Laboratory, Summit, N. J., and the Department of Chemistry, University of Texas.

It is now known that the rat requires at least 4 specific factors of the vitamin B complex: thiamin, riboflavin, vitamin B_6, and pantothenic acid. Only recently has the structure of pantothenic acid been determined[1, 2] and its synthesis accomplished,[3, 4] but the need for this factor in animal nutrition is already fairly well established.

The identification of the chick antidermititis factor as pantothenic acid was established independently by Woolley, Waisman and Elvehjem[5, 6] and Jukes.[7] Shortly thereafter, studies were undertaken by several groups of investigators to determine whether or not pantothenic acid was an essential factor in the nutrition of the rat. Subbarow and Hitchings[8, 9] concluded that pantothenic acid was probably one of the active factors in their liver filtrate preparations which were necessary for rat growth. El Sadr and his associates[10] also reported great similarity between their liver filtrate factor and pantothenic acid. Hoffer and Reichstein[11] demonstrated a rat-growth response to beta-alanine which these investigators suspect to be the active principle of the whole pantothenic molecule. Oleson, Woolley, and Elvehjem[12] found that crude preparations of pantothenic acid produced a growth response in rats fed a filtrate factor deficient diet.

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[1] Williams, R. J., and Major, R. T., *Science*, 1940, **91**, 246.

[2] Stiller, E. T., Keresztesy, J. C., and Finkelstein, J., *J. Am. Chem. Soc.*, 1940, **62**, 1779.

[3] Williams, R. J., Mitchell, H. K., Weinstock, H. H., Jr., and Snell, E. E., *J. Am. Chem. Soc.*, 1940, **62**, 1784.

[4] Stiller, E. T., Harris, S. A., Finkelstein, J., Keresztesy, J. C., and Folkers, K., *J. Am. Chem. Soc.*, 1940, **62**, 1785.

[5] Woolley, D. W., Waisman, H. A., and Elvehjem, C. A., *J. Am. Chem. Soc.*, 1939, **61**, 977.

[6] Woolley, D. W., Waisman, H. A., and Elvehjem, C. A., *J. Biol. Chem.*, 1939, **129**, 673.

[7] Jukes, T. H., *J. Am. Chem. Soc.*, 1939, **61**, 975.

[8] Subbarow, Y., and Hitchings, G. H., *J. Am. Chem. Soc.*, 1939, **61**, 1615.

[9] Hitchings, G. H., and Subbarow, Y., *J. Nutrition*, 1939, **18**, 265.

[10] El Sadr, M. M., Hind, H. G., Macrae, T. F., Work, C. E., Lythgoe, B., and Todd, A. R., *Nature*, 1939, **144**, 73.

[11] Hoffer, M., and Reichstein, T., *Nature*, 1939, **144**, 72.

[12] Oleson, J. J., Woolley, D. W., and Elvehjem, C. A., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 151.

They observed, further, that the pantothenic acid would not completely replace the original liver extract or crude filtrate preparations, thus showing the need for still another substance which they have called factor W. These investigators believe that the rat requires the intact pantothenic acid molecule since they could demonstrate no significant weight response in rats given beta-alanine, a constituent of pantothenic acid. Recently Unna[4] found that a single dose of 800 γ of synthetic d-pantothenic acid produced a rapid and striking gain in weight in rats depleted of pantothenic acid. The same dose of the l-form had no significant effect.

"Hydroxypantothenic acid" [N-(alpha-hydroxy-β,-β'-dimethylolbutyryl)-β-alanine] was recently synthesized by Mitchell, Snell and Williams[13] and was found to possess marked biological activity which varied not only with the microörganism but also with the conditions under which it was grown. Because of the variable results obtained with hydroxypantothenic acid and the concordant results obtained when concentrates from natural sources were tested for pantothenic acid, these investigators concluded that natural pantothenic acid is probably a single substance and that this hydroxypantothenic acid probably does not occur in such concentrates. Because of the striking biological activity of hydroxypantothenic acid in microörganisms, studies were undertaken to test the physiological activity of this compound in rats. The present publication reports the results of such a study.

Method. A litter of 13 rats was weaned at 35 days of age and given the following basal diet used by Elvehjem and his associates:[12] sucrose 76%; purified casein 18%; salts 4%, and corn oil 2%. In addition, each rat received 24 γ thiamin, 40 γ riboflavin, 50 γ vitamin B_6; 6 mg choline; 6 mg nicotinic acid; 6 mg vitamin C; and 200 γ vitamin K every other day and 35 mg halibut liver oil; 21 mg alpha tocopherol; and 350 mg linseed oil per week. At the end of 6 days, the 13 rats were divided into 4 groups. Group I (4 rats) served as control; Group II (3 rats) received 293 γ racemic synthetic Na pantothenate every other day; Group III (3 rats) received 720 γ racemic synthetic hydroxypantothenic acid every other day; Group IV (3 rats) received a mixture of 293 γ synthetic Na pantothenate and 720 γ synthetic hydroxypantothenic acid every other day. The study was continued over a period of 8 weeks. Weights were recorded on each animal 3 times a week.

Results. Table I shows the total increase in weight in each rat during the 8-week period of experimentation.

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[13] Mitchell, H. K., Snell, E. E., and Williams, R. J., *J. Am. Chem. Soc.*, 1940, **62**, 1791.

TABLE I.

Group I (Control)	Group II (Na pantothenate)	Group III (Hydroxypantothenic acid)	Group IV (Na pantothenate plus hydroxypantothenic acid)
♂ 49 g	♀ 95 g	♀ 61 g	♀ 110 g
♂ 56	♀ 89	♂ 78	♂ 78
♀ 30	♂ 121	♀ 62	♂ 115
♀ 20			

It can be seen from the data in Table I that the rats which received either synthetic Na pantothenate or synthetic hydroxypantothenic acid demonstrated a greater growth response than did control rats which received all of the known vitamins other than pantothenic or hydroxypantothenic acid. The growth response was greater in the Na pantothenate group than in the hydroxypantothenic acid group, indicating that although hydroxypantothenic acid has a stimulating effect, it is not a complete substitute for Na pantothenate in the nutrition of the rat. The two acids do not appear to supplement one another.

Summary. Rats maintained on a basal sucrose diet supplemented with vitamin B_1, riboflavin, vitamin B_6, choline, nicotinic acid, vitamin C, vitamin K, halibut liver oil, alpha tocopherol, and linseed oil exhibit a definite growth response following the addition of synthetic Na pantothenate or hydroxypantothenic acid. The growth response is greater in rats receiving Na pantothenate than it is in rats receiving hydroxypantothenic acid, indicating that Na hydroxy pantothenate is an incomplete substitute for Na pantothenate.

Thanks are due Research Corporation, New York, for a grant from the Williams-Waterman Fund for the Combat of Dietary Diseases in support of the animal work.

11751 P

Physiology of a Pure Culture of *Trichomonas vaginalis.* I: Population in Relation to pH.*

GARTH JOHNSON. (Introduced by T. L. Jahn.)
From the Departments of Obstetrics and Gynecology and Zoology, State University of Iowa, Iowa City, Iowa.

The effect of pH upon the growth of *Trichomonas vaginalis* is probably related to the problem of clinical control of the organism in cases of natural infection. This paper concerns the extremes of pH which will support growth *in vitro* and the optimum pH for the multiplication of the organism in bacteria-free culture.† [1]

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* The author wishes to thank Dr. T. L. Jahn whose advice has made this work possible, and Mr. Ray E. Trussell who aided materially in getting the work under way.

The culture medium used was Difco liver infusion agar slants overlaid with 5% human serum in modified Ringer's solution sterilized by filtration.‡ The pH of both the agar slants and fluid medium was adjusted with N/1 HCl and buffered with 0.25% sodium phosphate. The incubation temperature was 37.5 ± 1°C. The pH determinations were made with a glass electrode which was checked against two standard buffers.

The organisms were counted in a hemocytometer after 2, 4, and 6 days of incubation. Each count included 10 large squares, having a total volume of 1 cu mm. Six cultures were examined at each pH except in those cases where extraneous circumstances reduced the number to 5. Only minor shifts in pH were observed during incubation and the experimental cultures did not vary markedly from the uninoculated control tubes. The greatest difference between

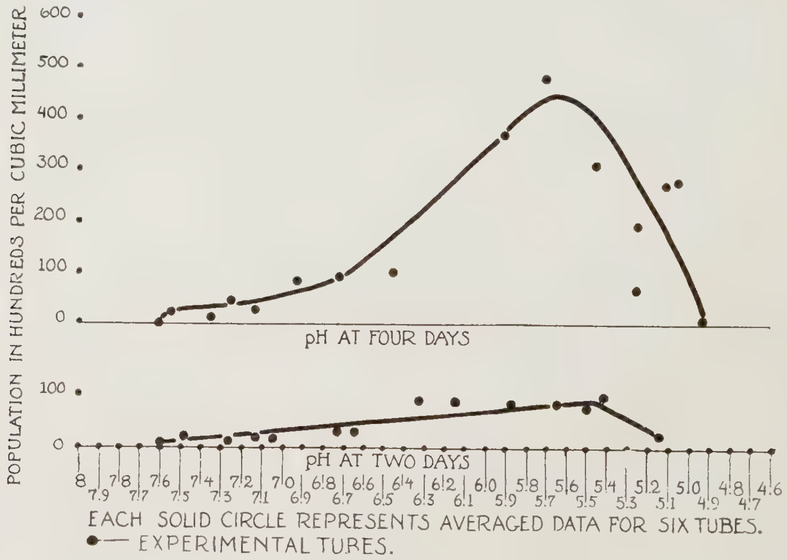

CHART I.

Explanation of Chart for Series II, Two and Four Days.

The pH of the cultures at the time of counting is plotted on the abscissa. The population in hundreds per cubic millimeter is shown on the ordinate. The curve for four-day cultures is concave in the upper ranges where the shift in pH in both experimentals and controls was away from the optimum.

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† The pure culture used in these experiments was isolated by Mr. Ray E. Trussell who kindly placed it at the author's disposal.

[1] Trussell, Ray E., *J. Iowa State Med. Soc.*, 1940, **30**, 66.

‡ Modified Ringer's solution: NaCl, 0.6 %; $NaHCO_3$, KCl, $CaCl_2$, 0.01 %.

experimental and control tubes was found to be 0.3 of 1 pH unit on the fourth day. Blood agar or chocolate agar plates were used to detect bacterial contamination at the conclusion of each experiment. Three cultures out of 305 used in 2 series and one subculture were found to be contaminated.

Optimum growth after 2 days' incubation occurred between pH 5.45 and 5.55, while after 4 days it was between 5.5 and 5.8. The highest pH supporting growth after 4 days was 7.55, the lowest 4.9. Two tubes out of 6 showed no live organisms at pH 4.9 after 4 days. Subculture on the sixth day revealed that the organisms had lost their viability above pH 7.55. (These data are taken from the second of 2 series of experiments with quite parallel results.)

11752 P

Alimentary Azotemia Due to Whole Blood Absorption from the Gastrointestinal Tract.

C. FRANK CHUNN AND HENRY N. HARKINS.
From the Division of General Surgery, Henry Ford Hospital, Detroit.

In the past six years numerous authors have reported an increase in blood urea nitrogen in certain cases of bleeding peptic ulcer. These include the reports of Sanguinetti,[1] Christiansen,[2] Meyler,[3] Alsted,[4] Sucic,[5] Schiff and Stevens,[6] and Stevens, Schiff, Lublin and Garber.[7] The chief theories to explain this increase were dehydration, shock, hepatorenal damage, hypochloremia, starvation and absorption of digested blood. Certain workers[2, 7] as well as Kaump and Parsons[8] believed that the last named factor (absorption of digested blood) is the most important. Our experiments tend to corroborate this conclusion.

Method. Citrated blood was given by stomach tube to 6 healthy dogs in 12 experiments (in 2 of these experiments dog blood and in the remainder beef blood was used). Urea nitrogen determinations by the method of Van Slyke and Cullen were made at least 5 times daily on blood from the jugular vein of each dog. After a control period of at least 24-hours which showed relatively little fluctuation, the dogs were given varying amounts of the beef or dog's blood by stomach tube and the blood urea nitrogen curve was continued.

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[1] Sanguinetti, Lucio V., *Arch. Argentinos de enfermedades de Aparato Digestivo y de la Nutrición*, 1934, **9**, 264.

[2] Christiansen, Tage, *Acta Med. Scand.*, 1935, **85**, 333.

[3] Meyler, L., *Acta Med. Scand.*, 1935, **87**, 313.

[4] Alsted, G., *Am. J. M. Sci.*, 1936, **192**, 199.

[5] Sucic, Dinko, *Klin. Wchnschr.*, 1935, **14**, 1316.

[6] Schiff, L., and Stevens, R. J., *Arch. Int. Med.*, 1939, **64**, 1239.

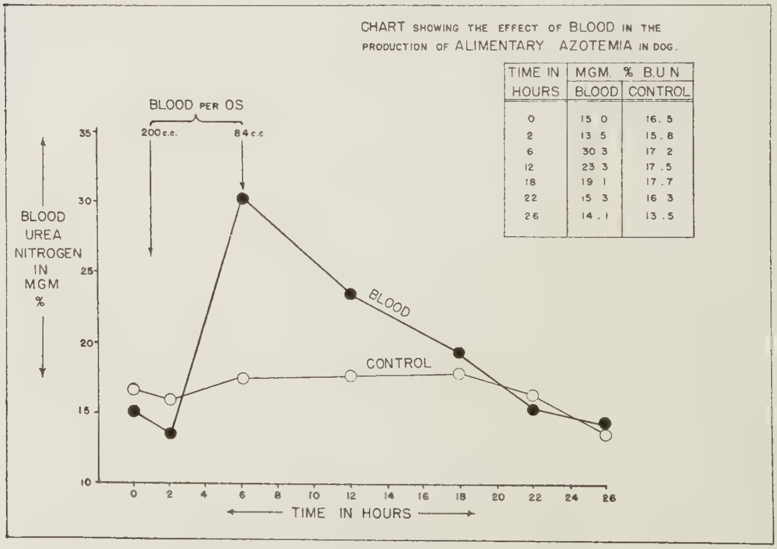
[7] Stevens, R. J., Schiff, L., Lublin, A., and Garber, E. S., *J. Clin. Invest.*, 1940, **19**, 233.

[8] Kaump, D. H., and Parsons, J. C., *Am. J. Dig. Dis.*, 1940, **7**, 191.

Results. In from about 3 to 7 hours after the administration of usually 200 cc of citrated blood by stomach tube, a definite elevation of the blood urea nitrogen occurred. A typical rise is shown in Fig. 1. Within 24 hours of the administration of blood the curve had usually returned to normal. In all instances a rise occurred and it was much greater than variations in the controls.

Comment. The clinical significance of this increase in blood urea nitrogen derives from the importance of its cause. If in a clinical case with bleeding peptic ulcer it is due to severe renal damage, its presence would tend to argue against operation. If on the other hand it is due to the mere presence of a quantity of blood in the gastrointestinal tract its occurrence would if anything favor operation. The present studies indicate that the second of these possibilities is actually the case.

Conclusions. (1) Intragastric administration of whole blood in



TIME IN HOURS	MGM. % B.U N	
	BLOOD	CONTROL
0	15 0	16. 5
2	13 5	15 . 8
6	30 3	17 2
12	23 3	17 . 5
18	19 1	17 . 7
22	15 3	16 3
26	14 . 1	13 . 5

FIG. 1.

The effect of citrated whole beef blood administration by stomach tube in the production of alimentary azotemia in a normal dog. Portions of the curve for 24 hours before and after that shown displayed very little variation from the flat control level.

12 experiments on 6 dogs caused a uniform and considerable rise in the blood urea nitrogen. (2) The increase seemed due primarily to absorption of digested blood. No evidences of starvation, dehydration, anemia, hypochloremia or shock were present in these dogs to explain the phenomenon. (3) It seems logical to assume that much of the rise in blood urea nitrogen observed in clinical cases of bleeding peptic ulcer is due to the same phenomenon, the resultant azotemia being of the alimentary type.

11753

Influence of Sex Hormones and Desoxycorticosterone on Melanophore Differentiation in Birds.

HOWARD L. HAMILTON. (Introduced by B. H. Willier.)

From the Biological Laboratories, University of Rochester, Rochester, N. Y.

Recently, it was found that explants of skin ectoderm from embryos of New Hampshire Red fowl occasionally yielded black melanophores, but red ones differentiated rarely, and a majority of the cultures were entirely negative. Since an examination of regenerating feathers from the adult (in which the plumage is predominantly red) showed that there was an abundance of red melanophores, it seemed desirable to find out what factors influence the differentiation of these pigment cells. The purpose of this report is to summarize some of the results which have been obtained by using sex hormones and a hormone of the adrenal cortex.*

In these experiments, a piece of skin was removed from the dorsal midline of a 6-day chick embryo (New Hampshire Red or Rhode Island Red) and divided into bilateral halves. One of these was explanted into a clot made up of embryonic extract mixed with plasma containing the sex hormone, and a control culture was made of the other half. Crystalline hormones were shaken up in blood plasma. Sesame oil, olive oil, and hormones dissolved in them were emulsified with plasma. Concentrations of hormones ranged from 20 to 300 γ per cc.

Red melanophores differentiated in cultures treated with sex hormones within 24 to 48 hours, and were similar to those in the

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* I am indebted to Professor B. H. Willier and Dr. R. K. Burns for suggestions in connection with this work, and to Dr. G. Stragnell of the Schering Corporation for his interest and for generous supplies of Oreton and Cortate.

TABLE I.

Summary of the Action of Sex Hormones, Sesame Oil, and Olive Oil on the Differentiation of Red Melanophores.

Test substance	No. of embryos sampled	Total No. of treated explants	% of explants containing red melanophores: Treated explants	% of explants containing red melanophores: Control explants
Estradiol dipropionate in sesame oil	47	148	37.8	6.0
Estradiol monobenzoate in sesame oil	10	19	63.2	10.0
Estrone (cryst.)				
Sample 1	4	13	30.8	0
Sample 2	21	43	23.3	13.6
Testosterone propionate in sesame oil	13	30	60.0	0
Sesame oil	37	73	31.5	2.6
Olive oil	10	38	23.7	15.8

regenerating feathers of adult birds. They were slightly smaller than black melanophores, and contained sub-spherical, orange-red granules which partially dissolved in alcohol or xylol after the cells were fixed. The rapid, almost vibratory, movement of these granules within the living cell indicated that the cytoplasm must be extremely fluid. Black melanophores contained dark-brown or black, rod-shaped granules which moved very slowly in living cells and were insoluble in alcohol and xylol. They differentiated *in vitro* at approximately the same time as the red melanophores. The percentages of explants which contained red melanophores due to the action of sex hormones are summarized in Table I.

Testosterone was far more potent in its action on melanophores than any of the female hormones. It not only affected 60% of the cultures, but also caused differentiation of a greater number of red melanophores in each separate explant.

The data show that sesame oil had a marked influence on the differentiation of red melanophores, but in every case solutions of hormones in the oil were much more active, so that there can be no doubt that the hormones had an action of their own. Furthermore, crystalline hormones, though practically insoluble in plasma, produced a measurable effect.†

Explants of skin from embryos of the New Hampshire Red, White Leghorn, and Barred Rock breeds of fowl were grown in clots containing the cortical hormone of the adrenal gland, desoxycorticosterone acetate (Cortate), in concentrations of 50 to 333 γ per cc.

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† The cause of the discrepancy in the results obtained from the two samples of estrone is not known. The preparations were obtained from two different sources and apparently differed only in the much smaller size of the crystals in sample 1.

No melanophores of any kind differentiated in treated explants from New Hampshire Red embryos, even though there were occasional cases in which red melanophores appeared in the corresponding control cultures. Similarly, all hormone-treated White Leghorn explants were entirely negative, although the controls contained the usual numbers of black melanophores which are short-lived though capable of synthesizing and depositing melanin granules. In the case of the Barred Rock, which possesses a relatively more viable pigment cell, the result varied with the age of the explanted tissue. When skin was taken from a 6-day embryo or younger, no differentiated melanophores were present at the time of explantation, and few or none of the melanoblasts developed pigment granules in the presence of the hormone. Skin from 7-day embryos or older already contained many black melanophores at the time of isolation. When it was grown in the corticosterone-plasma the melanophores which were present retracted their processes, rounded up, and died, and new ones were not differentiated to take their places. The reaction was not always complete; occasionally, expanded melanophores persisted in older explants or were newly differentiated in younger ones, but there were never many of them. Control cultures always contained great quantities of black, expanded melanophores.

The action of the cortical hormone, then, is apparently that of a suppressor of melanin formation, which causes degeneration of melanophores and inhibits the differentiation of new ones. This result may be of interest in view of the fact that clinically it is observed that Addison's disease is accompanied by a darkening of the skin as though the normal inhibitor of pigment formation had been removed with the destruction of the adrenal cortex.

11754

Activation of Pitressin by Acetic Acid.*

Eric Ogden and Leo A. Sapirstein.

From the Division of Physiology, University of California Medical School.

In the process of development of a technic for the extraction of pitressin from tissues as a preliminary to its assay, the effect of acetic acid on pitressin *in vitro* was studied.

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* This work was assisted by the John and Mary Markle Foundation and the Board of Research of the University of California.

TABLE I.
Activation of Pitressin by Acetic Acid.

Preparation (1)	Dose given, M.U. Treated (2)	Dose given, M.U. Control (3)	Dose assayed, M.U. Treated (4)	Dose assayed, M.U. Control (5)	Activation factor Col. 4/Col. 2 (6)	Activation factor Col. 4/Col. 5 (7)
M.U.						
150 boiled c* 50 cc 1% acetic acid	13.95	—	45	—	3.2	—
1000 ” c 10 ” ” ” ”	17.12	16.16	30.5	12.0	1.7	2.5
400 refluxed 15 min c 25 cc 1% acetic acid	4.68	4.76	8.1	11.4	1.7	0.71
400 ” ” ” ” ” ” ” ” ”	4.52	4.56	9.6	5.4	2.1	1.8
400 ” ” ” ” ” ” ” ” ”	8.30	8.76	13.2	8.3	1.6	1.6
4000 ” ” ” ” 20 ” ” ” ”	8.52	8.20	11.7	7.4	1.4	1.6
4000 ” ” ” ” ” ” ” ” ”		Pressor Assay			1.6	1.6
4000 ” ” ” ” ” ” ” ” ”	8.00	7.98	15.3	9.0	1.9	1.7
5000 ” 10 ” ” 25 ” ” ” ”		Pressor Assay			1.6	1.5
4000 ” 15 ” ” ” ” ” ” ”		” ”			1.7	1.3
1300 mixed cold c 10 cc 1% acetic acid	13.57	13.10	9.0	10.8	0.66	0.83
400 refluxed 15 min c water	4.01	3.99	1.5	5.8	0.37	0.26
4000 ” ” ” ” .1 N HCl		Pressor Assay			0.5	0.35
4000 ” ” ” ” .15 N Sod. Acetate		” ”			0.8	0.8

The antidiuretic assays (Burn)[3] were made on a newly calibrated group of rats and doses of treated pitressin were alternated with doses of untreated pitressin.

The pressor assays were made by intravenous injection into deeply chloretonized dogs of alternate similar doses of treated and untreated pitressin (Kamm).[4] The activation factor in these instances was obtained from the ratio of the maximum blood pressure rise obtained with the control and treated substances (Column 7), and checked by a ratio of the areas under the pressure curves (Column 6).

Columns (2) and (3) are in doses per rat. The paired doses are identical on a weight basis.

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*c = with.

[3] Burn, J. H., *Quart. J. Pharm.*, 1931, **4**, 517.

[4] Kamm, O., *J. Am. Chem. Soc.*, 1928, **50**, 573.

Table I shows the result of the assays of a variety of preparations.† Note in every case that the pitressin which had been boiled with acetic acid appears to have a definitely more potent effect than untreated pitressin, but that the other treatments involved a loss of potency. Column 1 indicates that this activation is a result of boiling with acetic acid rather than of boiling, concentration or acetate ions alone. The close similarity of the activation as measured by the antidiuretic method and by the pressor method is to be noted, though this does not necessarily indicate that the two effects (antidiuretic and pressor) are due to the same substance.

The failure of hydrochloric acid to activate pitressin (confirming Dale and Dudley[1] and others) suggests that the mechanism is not a hydrolytic one. The fact that the effect is observed equally with subcutaneous injection in the one assay and with intravenous injection in the other precludes the likelihood of the activation depending upon variations in the rate of absorption.

Noble, Rinderknecht and Williams,[2] observing potentiation of pitressin solutions assayed by the antidiuretic technic after treatment with certain heavy metal acetates, concluded that the effect of these augmenting substances depended on changes in the rate of subcutaneous absorption. The present investigation indicates the possibility that the observed augmentation may have been due to the effect of acetic acid derived from hydrolysis of the heavy metal acetates on the pitressin rather than to the effects of the salts on the rate of subcutaneous absorption.

Since acetic acid, which is commonly employed in the extraction of tissues for assay of pitressin content, is able to potentiate pitressin to a varying degree, there is a possibility that misleading results might be obtained by the application of this method unless extreme care is used in the standardization of all details such as the concentration of reagent and duration of procedures.

Summary. Pitressin when boiled with acetic acid becomes more potent as shown by subcutaneous antidiuretic assay and intravenous pressor assay. These effects are not due to boiling, concentration, acidity, or the presence of acetate ions. The significance of these findings in connection with the preparation of acetic acid extracts of tissues for pitressin assay is discussed.

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† These preparations were all made by dilution from pitressin generously given to us by Dr. Oliver Kamm of Parke Davis Laboratories to whom our thanks are extended.

[1] Dale, H. H., and Dudley, H. W., *J. Pharm.*, 1921, **18**, 1.

[2] Noble, Rinderknecht, and Williams, *J. Physiol.*, 1939, **96**, 293.

11755

Physiological Studies of Rhenium Compounds.

Frank Maresh, M. J. Lustok and P. P. Cohen. (Introduced by W. J. Meek.)

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A study of the behavior of potassium perrhenate in mice and rabbits[1] indicated that the element rhenium was not very toxic in the animal body. The low solubility of the $KReO_4$ often demanded injections of such large volumes that they exceeded the capacity of the animals. Since that time the more soluble salt $NaReO_4$ has been prepared. A study of some of its action in the animal body follows:

Toxicity in Rats. A $NaReO_4$ solution containing 42 mg of Re per cc of solution was injected intraperitoneally into 11 immature female rats weighing 58-71 g; the largest volume injected did not exceed 2.0 cc of solution. Six rats receiving 310, 350, 545, 574, 592, 600 mg of Re per kg of body weight lived without showing any visible symptoms; 3 rats receiving 830, 870, 890 mg of Re suffered from symptoms which vanished in a few hours or, at the latest, over night; 2 rats receiving 1050 and 1380 mg of Re died 60 and 30 minutes later respectively. The series places the lethal dose in the range 900-1000 mg of Re per kg of body weight. The small quantity of $NaReO_4$ available prevented the determination of this dose to a narrower range.

Immediately after an injection of the larger doses of rhenium the rats became pale and cyanotic. For at least an hour the respiratory rate and amplitude was increased, the respiration becoming laborious at times. A few minutes after the injection the tail and rear legs became rigid and extended, or they alternated between rigid extension and convulsions resembling somewhat those produced by strychnine. The convulsions could be induced by mild stimuli such as by tapping the cage, or by stamping on the floor. The front legs were never involved in the convulsions. The rats were able to right themselves but dragged their rear legs as they moved, propelled entirely by the front legs. The symptoms following a sublethal dose lasted for a few hours but disappeared completely and without showing any residual paralyses on the following morning.

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[1] Hurd, L. C., Colehour, J. K., and Cohen, P. P. Proc. Soc. Exp. Biol. and Med., 1933, **30**, 926.

Molybdenum. Because rhenium is associated with molybdenum minerals on the earth's crust, similar toxicity studies were conducted with molybdenum salts. Intraperitoneal injections of a sodium molybdate solution (25 mg Mo per cc) into 20 rats weighing from 140-260 g produced a lethargy. The rats curled themselves into a sphere, retained normal posture but refrained from any movement. As the duration of the action increased, the depth of the lethargy increased until the rats expired quietly in this state. The 10 rats receiving 47, 69, 73, 86, 91, 92, 95, 104, 113, and 114 mg Mo per kg weight showed a few transitory symptoms and remained well; the 10 rats receiving 117, 121, 137, 155, 156, 175, 178, 185, 245, and 350 mg Mo per kg weight died quietly within a few hours or over night. The lethal dose is between 114 and 117 mg Mo per kg weight or approximately one-eighth that of Re.

Other Rhenium Compounds. Solutions of the salts K_2ReCl_6 and $ReCl_3$, injected intraperitoneally into mice were toxic. These salts acted probably through the HCl liberated in the hydrolysis $K_2ReCl_6 + 2\ H_2O \rightleftarrows ReO_2 + 4\ HCl + 2\ KCl$ and $2\ ReCl_3 + 3\ H_2O \rightleftarrows Re_2O_3 + 6\ HCl$. Equivalent injections of HCl or $FeCl_3$ killed the mice in the same manner. A black precipitate of insoluble ReO_2 or Re_2O_3 covered the peritoneum at autopsy.

Blood Studies. Hammett[2] reported that subcutaneous injections of germanium dioxide (6.6 and 45 mg per kg of body weight) produced a polycythemia of 1-5 million cells lasting 10-14 days after the injection. In recent years other students[3] have demonstrated the erythropoietic action of cobalt. For a study of the erythropoietic action of rhenium, 6 male albino rats weighing 188-243 g, normal to all appearances, were placed on a Steenbock ration with ample water and good housing. From October 3 to October 21, (the control period) semi-weekly determinations of the weight, hemoglobin, erythrocytes, and leucocytes were made. From October 21 until December 16 aqueous solutions of $NaReO_4$ (40-230 mg of Re per kg of body weight) were injected subcutaneously at intervals of 2 or 3 weeks. Since the largest injection was about one-fourth of the minimal lethal dose, the rats did not exhibit any of the symptoms which appear with the large doses. Weekly determinations (and in 2 rats daily determinations) of hemoglobin, erythrocytes, leucocytes, weight and the general well being of the rats after the injections show that Re does not have any erythropoietic action and that the slight progressive decrease in erythrocytes and hemoglobin is

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[2] Hammett, F. S., Müller, J. H., and Nowrey, J. E., Jr., *J. Exp. Med.*, 1922, **35**, 173, 507.

[3] Orten, J. M., *Am. J. Physiol.*, 1936, **114**, 414.

TABLE I.

Date	Wt in g	Hemoglobin, g/100 cc blood	Erythrocytes in 10^6 per cu mm of blood
Oct. 3	210	13.4	10.5
" 14	243	13.8	7.5
" 19	259	16.9	8.1
" 21*	257	—	—
" 24	—	18.5	10.0
" 31	263	17.8	10.5
Nov. 9	271	14.8	8.4
" 13†	270	—	—
" 14	268	19.4	7.5
" 15	—	19.4	7.5
" 16	—	—	7.8
" 17	267	14.0	8.0
" 18	—	—	8.0
" 19	252	13.0	7.0
" 20	—	16.0	6.3
" 21	—	17.5	5.3
" 22	263	13.8	7.25
" 23	—	14.9	7.0
" 24	268	13.2	6.2
" 25	—	12.5	7.2
" 26	256	13.9	8.0
" 27	—	11.4	6.4
" 28	261	12.1	6.5

*Injected 10.9 mg Re per kg of rat intraperitoneally.
†Injected 58 mg Re per kg subcutaneously.

a secondary anemia due to frequent bleedings. A protocol for one rat receiving the largest amount of $NaReO_4$ appears in Table I.

Effects Upon Blood Pressure in Dogs. In a 5.5 kg dog under ether anesthesia and premedicated with morphine the mean femoral pressure rose from 108 to a maximum of 130 mm and returned to 108 mm after 5 minutes; the heart rate rose from 150 to 198 beats per minute after an intravenous injection of 25 cc of $NaReO_4$ solution containing 19 mg Re per cc. (86 mg per kg.)

An equal volume (25 cc) of normal saline solution injected intravenously both before and after the Re injection produced a temporary rise of 4 and 10 mm in blood pressure respectively.

In a 13.7 kg dog, the intravenous injection of 42 cc of $NaReO_4$ solution containing 20.4 mg Re per cc (62 mg per kg) increased the mean pressure from 118 to 144 mm for several minutes and decreased the heart rate from 102 to 96 beats per minute.

A 30 cc volume of normal saline solution injected intravenously into the preceding dog elevated the mean femoral pressure temporarily from 116 to 120 mm (4 mm), the heart rate changing from 96 to 102 beats per minute. The respiratory rate and amplitude were not disturbed by these injections of rhenium.

Discussion and Summary. Although about 20 of the rare elements have received some attention in pharmacology and some of these (thallium, gallium) have distinctive properties, rhenium surprises observers by its relatively low toxicity and general inertness in the body. The lethal dose for rats is of the order 900-1000 mg per kg of body weight. In rats it lacks the hemopoietic stimulus of Cobalt or Germanium, develops visible symptoms only in large doses, and produces only small transitory changes in blood pressure. Symptoms resembling the convulsions produced by strychnine suggest that the spinal cord has a high selectivity for the salts of Re.

We are indebted to L. C. Hurd for preparing and for supplying the $NaReO_4$ used in this report.

11756

Partition Studies on the Clot-Aiding and Related Blood Phospholipids.

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The phospholipid *cephalin* is a potent coagulation (thromboplastic) agent[1] and a natural blood constituent. Elucidation of its distribution and physicochemical state is needed to define its rôle in clotting mechanisms.

The coagulation defect in hemophilia is inadequately remedied by cephalin *(in vitro)*. Trypsin (thromboplastic enzyme[2]), however, restores the normal coagulation properties, especially under optimal conditions with respect to the calcium and phospholipid factors.[3] The cephalin content of the blood platelets, including hemophilic, has been investigated previously.[4] With the aid of recently developed micromethods,[5] the following data (Table I) have been

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* This research was carried out with the aid of a Sigma Xi grant.

[1] Ferguson, J. H., *Am. J. Physiol.*, 1938, **123**, 341.

[2] Ferguson, J. H., and Erickson, B. N., *Am. J. Physiol.*, 1939, **126**, 661.

[3] Ferguson, J. H., *Am. J. Physiol.*, 1939, **126**, 669.

[4] Erickson, B. N., Williams, H. H., Avrin, I., and Lee, P., *J. Clin. Inv.*, 1939, **18**, 81.

[5] Erickson, B. N., Avrin, I., Teague, D. M., and Williams, H. H., *J. Biol. Chem.*, 1940, **135**, 671.

TABLE I.
Phospholipid Partition of Plasma in Hemophilia.
(Mg per 100 cc plasma.)

	Total Phospholipid	Cephalin	Lecithin	Sphingomyelin
Normal*	189	55	99	35
Hemophilia	131	42	—	—
(5 patients)	165	45	66	55
	185	84	29	71
	170	49	59	61
	114	39	50	25

*Values from other studies.[5]

obtained on the partition of the plasma phospholipids (lecithin, cephalin and sphingomyelin) in hemophilia. There is no lack in the amount of the clot-aiding phospholipid cephalin.

Free and Bound Cephalin. It has been reported[2] that some prothrombin preparations although containing appreciable amounts of cephalin may be poorly (or not at all) activated by calcium alone. However, when similar amounts of pure cephalin solution are added, a highly active thrombin is formed. It is evident that the rôle of cephalin in this process is specifically dependent upon its physicochemical state or mode of combination. In the state of its natural protein combinations, cephalin does not seem to be available for direct participation in the coagulation rections. An attempt was made, therefore, to distinguish between the free and bound phospholipid in blood plasma.

A variety of methods was tried, including phospholipid analyses of plasma ultrafiltrates, protein-free filtrates, and ethyl ether and petroleum ether extracts of plasma and of plasma dehydrated with plaster of Paris. It was found practicable to recover a "free" phospholipid fraction by extraction with ethyl and petroleum ether after preliminary dehydration of plasma by pervaporation[6] in Visking casing sacs, exposed to warm air for 12 to 24 hours. This procedure was necessary for quantitative recovery (within ±4%) of pure cephalin from simple solution. Complete recovery of phospholipid in the presence of the plasma proteins always required the concomitant action of a denaturating agent, such as ethyl alcohol. The ready resolution and retention of clotting properties of pervaporated plasma (unextracted) support the belief that no significant denaturation and breakdown of the phospholipid-protein complexes occur during the cited procedures. The lipid-extracted dry plasma could be redissolved but had now lost its clotting properties.

The analytical data of Table II show the striking absence of all but traces (frequently less than the limits of the analytical methods) of "free" phospholipid from most normal and hemophilic plasmas. Positive findings were limited to the lipemias, occurring naturally in some hen plasmas and, as a clinical curiosity, on one human subject, *viz.*, a male child of 8 years,[7] who was known to have had this condition for 4 years without ever evincing any bleeding or clotting anomaly, according to routine clinical tests.

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[6] Thalhimer, W., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 230.

TABLE II.
Free Phospholipid of Citrated Plasma (Including Recovery of Added Cephalin) With and Without Trypsin.

Original sample	Free phospholipid (mg 100 cc plasma)	Cephalin added (mg 100 cc plasma)	Trypsin added (mg cc plasma)	Free phospholipid (mg 100 cc plasma)
Normal Human Plasma	0-0.2	—	—	0-0.2
		15	—	0.2-0.4
		30	—	1.4-5.7
		30	0.5	0.4
		30	2.0	2.9
		50	—	0.4-0.8
		50	2.0	1.1-1.4
		100	—	0.5-6.7
		100	2.0	2.6
Hemophilic Plasma	0-0.4	—	—	0-0.4
		—	0.1	0.4
		30	—	0.6-1.1
		30	0.5	0.5
		30	2.0	7.7
		85	—	2.3
		85	2.0	2.5-2.7
		100	—	1.1-1.9
		100	2.0	10.0
Dog Plasma	0-0.8	—	—	0-0.8
boiled	0.3-0.5	—	—	0.3-0.5
		—	0.1	0
		—	0.5	0.2
		—	2.0	0.3
Human Lipemic Plasma	31	—	—	31.
	31	—	2.0	42.
	87*	—	—	87.*
Hen Plasma	0.4-188†	—	—	0.4-188†
	0.4	—	3.0	0
	0.4	55	—	7.0
	0.4	55	3.0	5.4
	70.	55	—	90.
	188.	193	—	171.
	188.	193	4.0	346.

*12 mg of this consisted of cephalin.

†The amount of free phospholipid was related to the degree of the lipemia; of the 188 mg, 161 mg consisted of cephalin.

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[7] Bernstein, S. S., Williams, H. H., Hummel, F. C., Shepherd, M. L., and Erickson, B. N., *J. Pediat.*, 1939, **14**, 570.

On the addition of free cephalin to non-lipemic plasmas, including hemophilic, very small amounts could be recovered subsequently in the "free" form; nearly all of the phospholipid appeared to have been bound to the proteins.

The addition of crystalline trypsin,† in an effort to free the plasma phospholipid from its protein combinations, yielded variable results. There is no general confirmation of the preliminary data previously reported (on prothrombin solutions)[2] as suggestive of the ability of the proteolytic enzyme to liberate cephalin. Even with added cephalin or lipemic plasma, it was not possible to conclude whether the presence of trypsin modifies the proportion of free to bound cephalin.

Free and Bound Phospholipid in Platelet Lysates. Hemophilic platelets are not lacking in essential phospholipids[4] but platelet lysis is delayed in hemophilia.[8] The question of platelet lysis in relation to the liberation of phospholipid was investigated with the foregoing technic for free and bound phospholipid.

Platelets were recovered from citrated dog plasma by differential centrifugation to free them from plasma and red cells. A few leucocytes were doubtlessly included in the washed platelet residues. The platelet suspension was lysed with N/100 calcium chloride and the lysate removed after prolonged high-speed centrifugation. The residue was boiled in water and recentrifuged. In a first experiment the lysates were completely free from analyzable phospholipid. In a second experiment mere traces, *viz.*, 0.01 mg of "free" and 0.04 mg of "bound" phospholipid were found in the total lysate from the platelets obtained from 500 cc of dog blood. Additional traces were obtained from the extract of the boiled platelet residues. Both solutions, especially the latter, were fairly strongly thromboplastic in clotting tests on dog plasma.

The Phospholipid Content of Plasma Globulin and Albumin Fractions. Investigations of the phospholipids combined with the protein fractions of blood plasma were undertaken in view of the reported clot-aiding properties of the globulin[9] and the antithrombic potency of the albumin[10] fractions. Negligible amounts of phospholipid accompanied the globulins precipitated at pH 5.5 from dog, normal human, and hemophilic plasmas. Preliminary analyses of the globulin fraction precipitated with sodium sulfate, sodium sulfite, and ammonium sulfate led to the prompt discovery that the accompanying phospholipid varied from none to half of the total plasma phospholipid depending upon the salting agent employed. The results demonstrate the necessity of separating the individual protein fractions with minimal chemical alteration, before information can be secured concerning the protein-phospholipid complexes of blood plasma.

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† We appreciate the courtesy of J. H. Northrop for the supply of crystalline trypsin.

[8] Lee, P., and Erickson, B. N., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 264.

[9] Patek, A. J., and Taylor, F. H. L., *J. Clin. Inv.*, 1937, **16**, 113.

[10] Quick, A. J., *Am. J. Physiol.*, 1938, **123**, 712.

Summary. The phospholipid partition (lecithin, cephalin and sphingomyelin) of hemophilic plasma revealed no lack of the clot-aiding phospholipid cephalin.

A tested technic showed only minute traces of "free" phospholipid in normal and hemophilic plasma. Measurable amounts of uncombined cephalin and other phospholipids were found, however, in lipemic plasmas. Attempts to liberate cephalin by tryptic digestion of plasma were unsuccessful. Added cephalin combined with the plasma proteins to a large extent, except in lipemias.

The lysis of platelets in the presence of calcium (yielding an active thromboplastic solution) gave lysates containing little or no phospholipid, free or combined.

11757

Effect of Alpha-Tocopherol on Lesions of Skeletal Muscles in Rats on Vitamin A-Deficient Diets.

Cecil Krakower and Joseph H. Axtmayer. (Introduced by D. H. Cook.)

From the School of Tropical Medicine, San Juan, Puerto Rico.

Degenerative lesions of skeletal muscles have been described in rats on vitamin A-deficient diets.[1] We too have constantly observed such lesions in our experimental rats on a standard vitamin A-free diet.

In recent years muscular dystrophy in rats has been shown to be related to a deficiency in vitamin E. It was first produced in the suckling young of mother rats placed on a diet deficient in vitamin E by Evans and Burr[2] and the nature of the lesions in the muscles of the affected young were first described by Olcott[3] and recently by

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[1] Bessey, O. A., and Wolbach, S. B., "The Vitamins"—a Symposium, American Medical Association, 1939.

[2] Evans, H. M., and Burr, G. O., *J. Biol. Chem.*, 1928, **76**, 273.

[3] Olcott, H. S., *J. Nutrition*, 1938, **15**, 221.

Pappenheimer[4] and by Telford, *et al.*[5] Muscular dystrophy has also been described in older rats on vitamin E-deficient diets by Blumberg,[6] Ringsted,[7] Burr, Brown and Moseley,[8] Einarson and Ringsted,[9] Evans, Emerson and Telford[10] and Knowlton and Hines.[11] Some of these authors have given histological descriptions of the lesions.

While it has been demonstrated that the administration of vitamin E concentrates or wheat germ oil will prevent muscular dystrophy, Barrie,[12] Demole and Pfaltz,[13] and Goettsch and Ritzmann[14] have shown that alpha-tocopherol is similarly effective in preventing the disease in the suckling young and Knowlton, Hines and Brinkhous[15] in adult rats.

Inasmuch as the muscular lesions in rats on vitamin A-free diets resemble those of muscular dystrophy due to lack of vitamin E and as these vitamin A-free diets do not contain vitamin E, it was considered desirable to determine whether these muscular lesions were related to the deficiency of vitamin A or were due to lack of the vitamin E factor.

To avoid the use of an additional source of vitamin A in either wheat germ oil or its concentrates synthetic alpha-tocopherol* was employed. The standard vitamin A-free diet we have used consists of: Purified casein 18%, corn starch 68%, baker's yeast 10%, Osborne & Mendel's salt mixture 4% and viosterol 1 cc per kilo of diet.

In the first series there were 3 litters, the mothers of which had been previously fed Purina dog chow but were placed on the vitamin A-free diet 17, 14 and 14 days respectively postpartum. The young were weaned at 28 days and were henceforth continued on the vitamin A-free diet. There were 17 young in this series, 8 of these were fed 1.5 mg of alpha-tocopherol (Merck's) in olive oil weekly; the remaining 9 controls were fed olive oil alone. No cod liver oil supplement was given. Sixteen of the animals were killed when they were between 60 and 75 days of age. One rat died (63 days old) and was immediately autopsied. The initial weights at the time of weaning ranged from 33 to 57 g and the final weights between 55 to 89 g.

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[4] Pappenheimer, A. M., *Am. J. Path.*, 1939, **15**, 179.

[5] Telford, I. R., Emerson, G. A., and Evans, H. M., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 291.

[6] Blumberg, H., *J. Biol. Chem.*, 1935, **108**, 227.

[7] Ringsted, A., *Biochem. J.*, 1935, **29**, 788.

[8] Burr, G. O., Brown, W. R., and Moseley, R. L., Proc. Soc. Exp. Biol. and Med., 1937, **36**, 780.

[9] Einarson, L., and Ringsted, A., *Nutritional Abstracts*, 1938, **8**, 52.

[10] Evans, H. M., Emerson, G. A., and Telford, I. R., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 625.

[11] Knowlton, G. C., and Hines, H. M., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 665.

[12] Barrie, M. M. O., *Nature*, 1938, **142**, 799.

[13] Demole, V., and Pfaltz, H., *Schweiz. Med. Wchnschr.*, 1939, **69**, 123.

[14] Goettsch, M., and Ritzmann, J., *J. Nutrition*, 1939, **17**, 371.

[15] Knowlton, G. C., Hines, H. M., and Brinkhous, V. M., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 804.

* Kindly contributed by Merck & Co. and Hoffman-La Roche, Inc.

In the second series 2 litters with a total of 17 rats were employed. The mothers were maintained on Purina dog chow during gestation and lactation. The young were weaned at 28 days and placed on the vitamin A-free diet. Eight of these were given weekly injections subcutaneously of 5 mg d l alpha-tocopherol acetate (Hoffman-La Roche) in olive oil. The remaining 9 were left as controls. The diet of all animals was supplemented once or twice and in one instance 3 times with one or 2 drops of cod liver oil. All animals died at or were allowed to survive to ages ranging from 113 to 183 days. The animals that died were autopsied as soon as possible. The initial weights at weaning varied between 44 and 73 g and at death between 104 and 172 g.

Experimental and control animals were killed simultaneously when possible.

Results. In the first series (acute vitamin A deficiency) there were microscopic (but no macroscopic) muscular lesions in all control animals except the youngest one (60 days). These were of the character of hyaline necroses with or without reactive cellular changes and with little evident regeneration. The lesions were generally few but in some groups of muscle they were either absent or quite frequent. The most pronounced lesions in this series occurred in the oldest control rat (75 days).

In the experimental group that was fed tocopherol no muscular lesions were observed except a very rare hyaline necrosis in the muscles of the hind legs of one animal and of the forelegs of a second.

In the second series (chronic vitamin A deficiency) there were extensive muscular lesions in the control group, particularly the older ones (macroscopically clearly apparent only in one, due to calcification of the hyaline necroses). The lesions were as above with more active repair and regeneration.

In the experimental groups that received injections of tocopherol no lesions were observed except a single hyaline necrosis in the musculature of the forelegs of one animal and a rare lesion in the abdominal musculature of the second.

Many of the animals, particularly the older ones, whether control or experimental, exhibited varied degrees of weakness and some incoördination towards the latter part of the experimental period. The central and peripheral nervous systems were not examined. As might be expected the alpha-tocopherol had no effect upon either the extent or character of the characteristic lesions of vitamin A deficiency present in all animals, and testicular atrophy occurred as readily in the experimental as in the control animals. Focal areas of interstitial myocardial infiltration occurred as frequently in the one as in the other.

Conclusions. Muscular lesions hitherto described in vitamin A deficiency in rats can be prevented by the administration of alpha-tocopherol and hence are not related to the vitamin A-deficient state. Vitamin E or alpha-tocopherol should form a part of the basal diet used to produce vitamin A deficiency in rats.

11758

Histamine in the Rabbit Skin.

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Using the trypan blue test we have shown[1] that histamine injected intradermally in the very young rabbit does not cause a seepage into the injected area of the dye injected intravenously. This fact is consistent with an observation described by Feldberg and Kellaway[2] on the pharmacological effect of histamine on the cat. Very young cats are very resistant to the injection of histamine. In particular, the pressure in the pulmonary artery is only slightly altered by the intravenous injection of histamine in young cats, while the same dose injected into old cats may produce marked changes. By determining the histamine content of the lungs in cats varying in weights from 0.5 to 6 kg, they have shown a parallelism between the increasing amount of histamine in the lung tissue and the pharmacological effects produced by the drug when injected into the vein. This work was undertaken to ascertain whether a parallelism exists between the histamine content of the skin and the response to the trypan blue test.

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* Fellow of the John Simon Guggenheim Memorial Foundation.

[1] Rocha e Silva, M., *Nature*, 1940, **145**, 591.

[2] Feldberg, W., and Kellaway, C. H., *Aust. J. Exp. Biol. and Med. Sc.*, 1937, **15**, 81.

Experimental. We have determined (41 rabbits) the amount of histamine in rabbit skin by employing the Barsoum and Gaddum method[3] as modified by Code.[4] A technic devised by us[5] was used to reduce the volume of the extract, when desirable. All figures regarding histamine refer to histamine dihydrochloride which was used in the assays. Simultaneously with the collection of the fragment of skin for extraction we determined the area of the skin slices. A rectangle of the skin surface was drawn against cellophane paper and the area measured over millimetered paper. Then the skin fragment was cut out with sharp scissors, the subcutaneous connective tissue carefully dissected and the rectangle of the skin weighed. The skin fragments were removed from the skin of the abdomen near the midline; in the newborn the skin from the side of the abdomen was included. The weights of the fragments extracted ranged from 0.3 to 6.0 g and the areas from 10 to 60 sq cm.

For depilation of the area of the skin, the use of chemicals commonly employed has been avoided by using scissors to cut the hair close to the skin surface. Perhaps this practice explains the fact that our figures are lower than those appearing in previous publications.[6, 7, 8]

Results. The data in Fig. 1 show that immediately after birth the skin thickens and then becomes thinner and thinner until the body weight attains 300 to 600 g. In the adult rabbit (over 1500 g body weight) the skin is maximally thick. The variation in the histamine content is less since it decreases regularly from young rabbits to adult ones (Fig. 2). The determination of the curve of thickness of the skin as related to the body weight was necessary since one might be induced to interpret this decrease in the histamine content by assuming that the same quantity of histamine would be connected with a certain area and not with a certain weight of the skin. It may be seen, however, that very young rabbits, whose skin is as thick as that of adult rabbits, have large amounts of histamine in each gram of skin tissue.

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[3] Barsoum, G. S., and Gaddum, J. H., *J. Physiol.*, 1935, **85**, 1.

[4] Code, C. F., *J. Physiol.*, 1937, **89**, 257.

[5] Rocha e Silva, M., *Nature*, 1939, **144**, 252.

[6] Tarras-Wahlberg, B., *Klin. Wochnschr.*, 1937, **16**, 958.

[7] Riesser, O., *Arch. f. exp. Path. u. Pharm.*, 1937, **187**, 1.

[8] Rocha e Silva, M., and Bier, O., *Arq. Inst. Biol.*, 1938, **9**, 133; *Compt. Rend. Soc. de Biol.*, 1938, **129**, 1140.

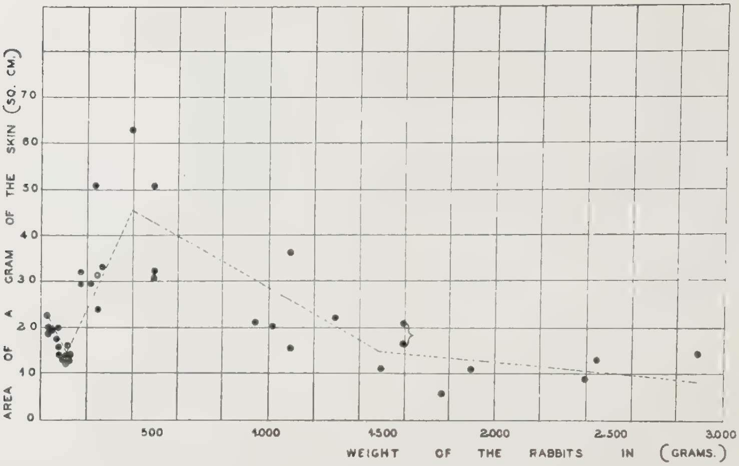

Fig. 1.
Curve of thickness of the skin of rabbits of different weights.

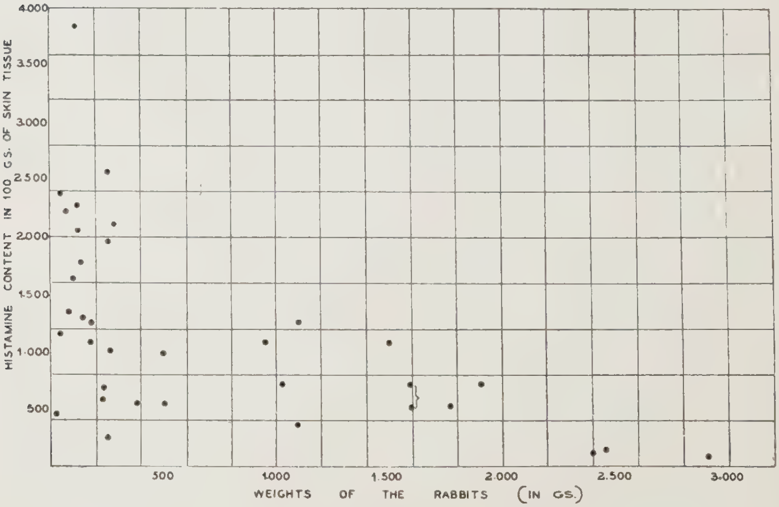

Fig. 2.
Histamine content of the skin in rabbits of different weights.

Hence, it is clear that the parallelism found by Feldberg and Kellaway[2] between the pharmacological action of histamine and the amount of histamine present in the lung tissue does not hold in the case of rabbit skin. Adult rabbits, which react very well to histamine intradermally injected by showing a very beautiful blue spot after intravenous injection of trypan blue, have per gram weight of skin a very low histamine content in their skin, while the histamine content is sometimes exceptionally high in the skin of very young rabbits which react poorly or not at all to the intradermal injection of histamine.

Whether this high amount of histamine in the skin of very young rabbits bears some relationship to the physiology of the skin is an open question.

11759

Influence of Nutritional Intake Upon Concentration of Vitamin A in Body Tissues.

Hsueh-Chung Kao and H. C. Sherman.

From the Department of Chemistry, Columbia University, New York City.

Batchelder's finding[1] that systematically increased intakes of vitamin A resulted in progressively more vigorous offspring even beyond the level at which the parents had reached the plateau of outwardly apparent wellbeing, suggests that liberality of intake of vitamin A may be a significantly influential factor in the body's internal environment, and adds importance to the question how generally the concentration levels in the body are influenced by the level of intake.

That the vitamin A content of the liver varies widely with the level of intake has been universally recognized since the quantitative work of Baumann, Riising, and Steenbock.[2] More recently Steininger, Roberts, and Brenner[3] have investigated the fluctuations of the vitamin A concentration in the blood. Are there corresponding fluctuations in the muscles? Conclusive experimental demonstration is difficult because the concentration of vitamin A in the muscles is so low. Boynton's bioassays[4] indicated that muscle contained only 0.25% to 0.5% of the concentration of vitamin A found in the livers of the same animals. Baumann, Riising, and Steenbock[2] reported "negative results" from their tests for vitamin A in the blood and muscles of normally nourished rats, but also spoke of their findings as in harmony with those of Boynton.

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[1] Batchelder, E. L., *Am. J. Physiol.*, 1934, **109**, 430.

[2] Baumann, C. A., Riising, B. M., and Steenbock, H., *J. Biol. Chem.*, 1934, **107**, 705.

[3] Steininger, G., Roberts, L. J., and Brenner, S., *J. Am. Med. Assn.*, 1939, **113**, 2381.

[4] Sherman, H. C., and Boynton, L. C., *J. Am. Chem. Soc.*, 1925, **47**, 1646.

The present paper reports the results of a reinvestigation of the vitamin A contents of the livers and muscles of rats receiving diets of different vitamin A values. These rat tissues were assayed by the single-feeding method first worked out by Todhunter,[5] and now further developed to include the use of side-by-side controls receiving systematically graded amounts of the official U.S.P. reference cod liver oil, and the statement of the data in terms of International Units. The positive control animals, to the numbers of 17, 19, 29, and 22, respectively, were fed 7.5, 15, 30, and 45 International Units each in a single dose. The corresponding measured areas representing the gains in body weight and length of life over the negative control animals were 138, 254, 368, and 567, respectively. By the use of these quantitative data, the results of feeding the parallel test animals with weighed amounts of the tissues under investigation become quantitative measures of the vitamin A concentration in terms of International Units per gram. We have further verified the findings by making use both of simple direct interpolation and of the "curve of response".

Like Steenbock and coworkers,[2] we find that a predominant proportion of any *surplus* intake is stored in the liver, so that an increment of intake may result in a greater than arithmetically proportionate increase in the concentration of vitamin A in the liver. Thus in typical experiments, they found that doubling the intake increased the concentration in the liver about five-fold (from 23 to 105 "Blue units"); and we find that raising the level of intake about four-fold results in a ten-fold increase of concentration in the liver, *viz.,* from 9 I.U. to 96 I.U. per gram, and in some of our experiments a still more marked increase. Further details of our data on liver may be omitted to economize space, inasmuch as our findings are here so largely confirmatory of the Wisconsin work.

In the case of muscle, while the low concentration makes quantitative measurement and interpretation of vitamin A values quite difficult, the discrepancy, between the "negative" findings at Wisconsin and the measurements previously made by Boynton, calls for fuller consideration of our present data. In 4 series of experiments comparing the tissues of animals reared on each of 2 adequate diets containing 4 to 5 I.U. of vitamin A per gram of dry food and on the same diets enriched to a value of 16 to 20 I.U. per gram by the addition of cod liver oil, and making such comparisons on both 30-day-old and 60-day-old rats, the average measurements summarized in Table I were obtained. As all these figures are near the limit of accuracy attainable in any individual measurement, there

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[5] Sherman, H. C., and Todhunter, E. N., *J. Nutrition,* 1934, **8**, 347.

TABLE I.
Concentrations of Vitamin A Found Per Gram of Muscle.

Description	From rats which had received:			
	Basal diet		Enriched diet	
	(No. of cases)	Mean value I.U.	(No. of cases)	Mean value I.U.
"Diet 13" rats at 30 days	(6)	0.0	(6)	0.4
" 132 " " 30 "	(6)	0.3	(7)	0.4
" 13 " " 60 "	(6)	0.3	(6)	0.4
" 132 " " 60 "	(7)	0.2	(6)	0.4
Average of the 4 series	(25)	0.2	(25)	0.4

may be room for difference of scientific opinion in their interpretation. In our opinion, the strongest probability is that the very low concentration of vitamin A in muscle is influenced by the nutritional intake. Any one of the 4 comparisons (Table I) standing alone might well be regarded as inconclusive; but in view of the fact that there is a difference in each of the 4 series and always in the same direction, and the further fact that in the average of the 4 series comprehending 25 individuals each on basal *versus* enriched diet there appears to be twice as much in the muscles of the animals which had received the higher intake, it seems to us distinctly more probable that this difference is significant than that it is merely accidental.

It is consistent with previous knowledge of the distribution of vitamin A in the body that the responses of blood-concentration to intake should influence the muscle-concentration in the same direction, even though in lesser degree. It is also consistent that a four-fold increase of intake should cause a more than four-fold increase of concentration in the liver and a less than four-fold concentration in the muscles.

Thus there is a consistent trend of evidence of different kinds bringing cumulative support to the view that healthy muscle contains a small amount of vitamin A as a normal constituent and that the concentration of vitamin A in the muscle is in some measure influenced by the level of nutritional intake.

Other researches in this laboratory seek to extend this field of investigation by experiments from different dietary starting points and by the development of improved methods of measuring the concentration of vitamin A in the tissues.

The assistance of grants from the Rockefeller Foundation and from the Carnegie Corporation of New York through the Carnegie Institution of Washington is gratefully acknowledged.

11760 P

Production of Experimental Jejunal Ulcer.

M. J. Schiffrin. (Introduced by B. P. Babkin.)

From the Department of Physiology, McGill University, Montreal, Canada.

The following method has been devised for the production of experimental jejunal ulcer. The abdomen of a cat, under Nembutal anaesthesia, is opened and 3 adjacent segments, each 6-8 cm long, are cut from the first loop of the jejunum distal to the ligament of Treitz. A mesenteric pedicle of adequate size is chosen to ensure a good blood supply to each segment. L-shaped cannulae with rounded tips are tied into the ends of each segment. The sections of intestine are then replaced in the abdominal cavity, care being taken to avoid strangulation. The open ends of the cannulae are brought through stab incisions in the abdominal wall, which is then closed.

The solutions to be perfused through the segments are pre-heated to 37-38° C. and are introduced via the distal cannulae to be drained proximally. A constant pressure of about 20 mm Hg is maintained

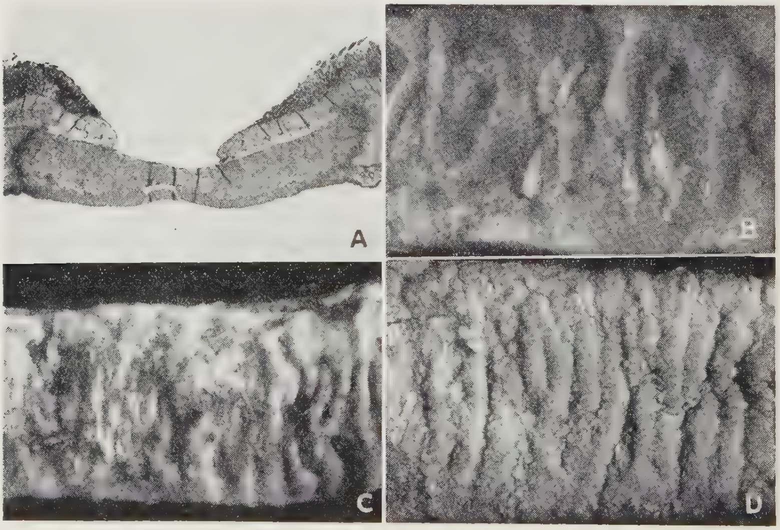

Fig. 1.

(a) Perfusion of jejunum with acid-pepsin (described in text). (b) Perfusion with 0.1 N HCl: little destruction of villi and some inflammatory changes. (c) Perfusion with acid-pepsin solution: upper left quadrant, perforation; lower right quadrant, ulceration through circular muscle; lower center, ulceration through mucous membrane; multiple ulceration marked by small blood clots throughout. (d) Perfusion with acid-pepsin and colloidal aluminum hydroxide: coating of aluminum hydroxide; no pathological changes. *Note:* b, c, and d are adjacent segments of jejunum from one animal and were each perfused for 12 hours.

by filling and inverting a 200-cc burette over a funnel leading to the heating jacket. The solutions used were (a) 0.1 N HCl, pH 1.2; (b) 3% pepsin (Parke, Davis & Co., 1:3,000) in 0.1 N HCl, pH 1.2; (c) 1 part colloidal aluminum hydroxide in 10 parts solution "b", pH 3.5; (d) 1 part aluminum phosphate gel in 10 parts solution "b", pH 2.0. The solutions are introduced at a rate of 1-2 cc per minute. After 12 hours, the sections of intestine are removed, examined grossly, and then fixed for histological study.

The experimental data are shown in Table I. In all but one instance, blood appeared in the acid-pepsin perfusate during the first 2 hours. Perforation occurred 3 times as a result of perfusion with acid-pepsin. This solution always caused ulceration, which usually consisted of 2 large areas of ulceration at the points of contact of the intestinal mucosa and the tips of the cannulae. There were moreover multiple small ulcers throughout the rest of the intestinal segment. Hydrochloric acid alone caused some destruction of the villi, but ulceration was never observed. The addition of either aluminum hydroxide or aluminum phosphate to the acid-pepsin mixture protected the intestine from damage.

The typical microscopic picture after 12 hours' perfusion with acid-pepsin is shown in Figure 1(a). Not only are the mucous membrane and the sub-mucosa destroyed, but part of the circular muscle as well. Both colloidal aluminum hydroxide and aluminum phosphate form a protective coating over the surface of the intestine and prevent ulceration. This is to be expected, since it has been shown in this laboratory that both compounds precipitate pepsin.[1, 2] This is in addition to their buffering action.

TABLE I.
Perfusion of Jejunal Segments.

Date, 1940	Sol. "a" 0.1 N HCl pH 1.2 cc/hr	Sol. "b" 3% pepsin in 0.1 N HCl pH 1.2 cc/hr	Sol. "c" 1 $Al(OH)_3$ in 10 sol. "b" pH 3.5 cc/hr	Sol. "d" 1 $AlPO_4$ in 10 sol. "b" pH 2.0 cc/hr	Duration hrs
August 28	81	82			10½
” 30	91	89	84		12
Sept. 2	85	88	89		12
” 3	120	120	111		10
” 4	96	96		96	5¼
” 5	108	105*		96	12
” 6	123	120		115	10
” 9	118	113		117	12
” 10	112	101*	92		7¾
” 24	96	96*	104		12

*Perforation of loop perfused with acid-pepsin solution.

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[1] Komarov, S. A., and Komarov, O., *Am. J. Dig. Dis.*, 1940, **7**, 166.

[2] Schiffrin, M. J., and Komarov, S. A., to be published.

This method of producing ulceration is similar to some described by other workers, but to the best of the writer's knowledge no acute experiments of this type have been extended over a period of 12 hours and no ulceration or perforation was previously observed. The extremely important rôle of pepsin in the etiology of ulcer is indicated. The mechanism by which aluminum hydroxide and aluminum phosphate protect the intestinal mucosa may be studied *in vivo* by this technic.

We intend to apply the same method of perfusion to other parts of the gastro-intestinal tract.

11761

Comparative Activity of Naturally Occurring Estrogens on the Infantile Rat Uterus and Vagina.* †

RALPH I. DORFMAN.

From the Laboratory of Physiological Chemistry and the Adolescence Study Unit, Yale University School of Medicine, New Haven, Conn.

In a previous report it was shown that there is a distinct difference between estrone and estriol in their respective abilities to cause hypertrophy of the infantile rat uterus and vaginal introitus.[1] These observations have been confirmed and extended by other workers to include estradiol.[2, 3] In this communication we have further extended these comparative studies to include, in addition to estrone, α-estradiol, and estriol, the benzoates of estrone and estradiol and also equilen and equilenin.

The estrogens employed in this study were dissolved in olive oil so that the daily amount administered was always contained in 0.2 cc of oil. Twenty-five-day old female rats were injected subcutaneously once daily for 5 days. Twenty-four hours after the last injection, the animals were sacrificed. Body weight, uterine weight, and vaginal

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* Aided by grants from the Committee on Scientific Research of the American Medical Association and the Rockefeller Foundation.

† Presented at the 31st annual meeting of the American Society of Biological Chemists, Memphis, Tenn., April, 1937.

[1] Dorfman, Ralph I., Gallagher, T. F., and Koch, F. C., *Endocrinology*, 1935, **19**, 33.

[2] Marlow, H. W., *Science*, 1936, **84**, 377.

[3] Lauson, H. D., Heller, C. G., Golden, J. B., and Sevringhaus, E. L., *Endocrinology*, 1939, **24**, 35.

TABLE I.

Estrogen	Daily dosage in μg	No. of animals	Uterine ratio*		Vaginal Introitus %
			Extremes	Mean	
0	0	10	0.49-0.72	0.65	0
α-Estradiol	0.01	5	0.60-1.0	0.85	40
	0.02	4	0.98-1.6	1.3	63
	0.06	4	1.80-2.3	2.1	100
	0.25	4	1.70-3.6	2.6	100
α-Estradiol-benzoate	0.07†	4	1.50-2.4	2.0	25
	0.14†	4	1.90-2.6	2.2	100
	0.29†	4	2.60-3.0	2.8	100
Estrone	0.14	5	0.92-1.2	0.99	30
	0.21	5	1.20-2.1	1.7	100
	0.39	5	2.20-7.8	5.1	100
Estrone-benzoate	0.14‡	7	0.60-0.79	0.70	0
	0.28‡	5	0.85-1.0	0.98	0
Estriol	0.55	5	0.76-1.0	0.86	60
	5.50	5	0.85-1.1	1.00	100
	16.50	5	1.00-1.2	1.10	100
	55.00	3	1.20-2.1	1.70	100
Equilin	0.17	5	0.99-1.5	1.2	70
	0.35	5	1.40-2.3	1.5	90
	0.50	4	2.60-3.2	2.8	100
Equilenin	0.17	5	0.57-0.70	0.64	0
	0.35	5	0.78-0.86	0.82	0
	0.50	5	0.71-1.30	0.99	20
	0.80	5	0.80-1.40	1.10	80
	1.00	5	1.10-2.20	1.60	100

*Uterine Ratio — $\frac{\text{Uterus in mg}}{\text{Body weight less gut in g}}$

†Represents weight of free estradiol.

‡ " " " " estrone.

introitus were determined. At each concentration for each estrogen 4 or more animals were used, except in one instance (estriol at 55γ per day) when 3 animals were employed.

The comparative uterine and vaginal activities of the estrogens studied are presented in Table I and Fig. I. α-Estradiol ranked as the most active estrogen in its ability to hypertrophy the uterus and produce vaginal introitus. As small a daily dose as 0.01 μg produced a 30% increase in uterine weight and 40% vaginal introitus. The descending order of uterine activity was α-estradiol, estrone, equilin, equilenin, and estriol. The descending order of activity on the vagina was α-estradiol, estrone, equilin, with estriol and equilenin being the least active of the group and of about the same magnitude.

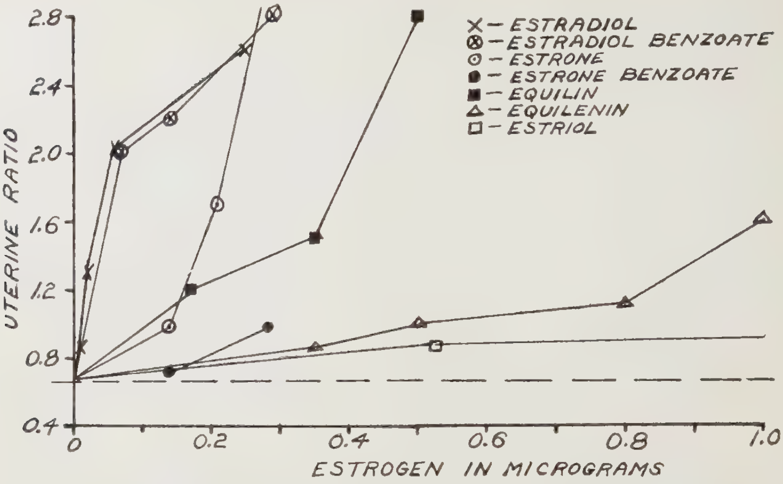

Fig. 1.

The comparative activity of naturally occurring estrogens on the infantile rat uterus. The Uterine Ratio represents the ratio of the uterus in mg to the body weight in g.

As previously described, the activity of estriol on the uterus is unique. Though a small dose stimulates the uterus, even a large dose (100γ) still does not hypertrophy the gland to its maximum size.

Under the conditions of the experiment, the uterine and vaginal activity of α-estradiol and its monobenzoate were of approximately the same magnitude, whereas estrone was definitely more active than its monobenzoate. The reason for this difference is not at the present apparent.

We are grateful to Dr. E. Schwenk of the Schering Corporation for the supply of estradiol and estrone, to Dr. O. Kamm of Parke, Davis and Company for the supply of estriol, and to Dr. Roussel of Paris for supplying us with equilen and equilenin.

11762 P

Respiration of Isolated Liver and Kidney Tissues from Adrenalectomized Rats.

S. R. Tipton. (Introduced by F. A. Hartman.)
From the Department of Physiology, The Ohio State University, Columbus.

After adrenalectomy the basal metabolism of rats is depressed. It is possible that the depression is the result of metabolic disturbance in certain special organs or tissues rather than a general metabolic weakness. The oxygen consumption of brain tissue is not influenced by removal of the adrenal cortex[1, 2] nor is that of skeletal muscle.[2] The hepatic and renal malfunction resulting after adrenalectomy suggests that energy reactions in these tissues may be altered.

The rate of oxygen consumption of liver and kidney slices from rats showing marked symptoms of insufficiency (about 9 days after operation) is significantly depressed. The mean values together with the standard error of the mean are shown in Table I. The tissues were studied in Ringer's solution with phosphate buffer at pH 7.3 with no added substrate. The depression is not due to the starvation resulting from the marked anorexia which is present since a group of starved normal rats showed values very similar to those of the well-fed normals.

TABLE I.

Q_{O_2} of Liver and Kidney Slices from Normal and from Adrenalectomized Rats.

Liver (20 rats)		Kidney (10 rats)	
Normal	Adrenalectomized	Normal	Adrenalectomized
Q_{O_2}	Q_{O_2}	Q_{O_2}	Q_{O_2}
8.68 ± 0.15	5.86 ± 0.12	18.66 ± 0.31	13.73 ± 0.45

The rate of oxygen consumption of liver slices from normal rats is considerably increased when pyruvic acid and succinic acid are added to the medium, indicating an active oxidation of these metabolites. It is now recognized that these substances are important intermediary metabolites in carbohydrate oxidation. The data presented in Table II show that after adrenalectomy the rate of oxidation of these substances is slower. Pyruvate oxidation is influenced to a greater extent than that of succinic acid (40% depression with pyruvate as compared with 30% in the case of succinate). The

TABLE II.

Q_{O_2} of Liver Slices from Normal and Adrenalectomized Rats in the Presence of Added Substrates.

	Control Ringer's	Sodium Pyruvate .04 M	Sodium Succinate .04 M	Dextrose .04 M
	Q_{O_2}	Q_{O_2}	Q_{O_2}	Q_{O_2}
Normal	8.68 ± 0.15	14.01 ± 0.53	17.27 ± 0.53	9.48 ± 0.21
Adrenalectomized	5.86 ± 0.12	7.13 ± 0.17	9.88 ± 0.34	6.38 ± 0.10

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[1] Tipton, S. R., *Am. J. Physiol.*, 1939, **127**, 710.

[2] Crismon, J. M., and Field, J., 2nd, *Am. J. Physiol.*, 1940, **130**, 231.

effect of adding dextrose to the medium is the same after adrenalectomy as before.

It is concluded that oxidative systems are impaired by a deficiency of the adrenal cortex hormones, but the weakening appears to be general in nature rather than restricted to some one component of the oxidation chain.

11763

Nicotinic Acid as a Growth Factor for *H. Pertussis.*

J. W. Hornibrook. (Introduced by R. E. Dyer.)
From the Division of Infectious Diseases, National Institute of Health, U. S. Public Health Service.

It has been shown that *H. pertussis* Phase I will grow in a liquid medium containing only soluble starch, hydrolyzed casein, cystine and salts.[1] Yeast extract was found to stimulate growth but was not essential. Recently it was found that an amino acid mixture could be substituted for the casein. If the inoculum were so small as to carry over no appreciable amount of growth substance from the Bordet Gengou slant, yeast extract was found to be essential for growth. On testing a number of known substances present in yeast, it was found that nicotinamid or nicotinic acid could be substituted for yeast.

To prepare the medium 2 solutions, A and B, are mixed, brought to a volume of 100 cc, adjusted to a pH of 7.4, tubed in 2 cc quantities and autoclaved.

Solution A. NaCl, 0.7 g; KCl, 0.02 g; CaC_{12} anhyd., 0.02 g; MgC_{12}, 0.01 g; Na_2CO_3, 0.05 g; KH_2PO_4 Mono., 0.025 g; NH_4Cl, 0.02 g; soluble starch, 0.1 g; water, 50 cc.

The ingredients are mixed, autoclaved, cooled and filtered through paper.

Solution B. d glutamic acid, 0.18 g; l tyrosine, 0.02 g; glycine practical, 0.0045 g; l proline, 0.04 g; l histidine, 0.01 g; d arginine, 0.02 g.

The above are mixed dry and to them is added 0.001 g cystine dissolved in a drop or two of 10 N HCl. 10 cc of H_2O is added and normal NaOH is added until the acids are in solution.

On incubation the tubes are slanted at an angle of approximately 6 degrees from the horizontal.

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[1] Hornibrook, J. W., *Public Health Reports*, 1939, **54**, 1847.

The inoculum, a saline suspension of organisms from a B.G. slant, was progressively diluted until no growth took place in the above medium. At this stage the addition of nicotinic acid or nicotinamid (0.5 to 0.001 γ per cc) would allow the organisms to grow.

The above medium is not complete as the addition of a small amount of hydrolyzed casein results in a heavier growth.

Summary. Nicotinic acid or nicotinamid favors the growth of Phase I *H. pertussis.*

11764

Effect of Diminishing Doses of Post-Pituitary Extract on Urinary Excretion of Water and Chlorides.

HERBERT SILVETTE. (Introduced by H. E. Jordan.)
From the Department of Pharmacology, University of Virginia.

Previous work has indicated that, when the dose of injected post-pituitary extract is progressively reduced, the excretion rates of water and chlorides were changed independently of one another.[1] The present experiments were designed to extend these observations by following the change in urinary excretion over a wide range of extract dosage. Thus the dose of extract was varied from 3000 milliunits per 100 g of body weight down to 2.5 milliunits. White rats were employed, weighing about 200 g. Each animal was used but once to prevent any possible complication due to sensitization

TABLE I.
Urine and Chloride Excretion After Various Doses of Post-Pituitary Extract.

			Avg 6-hour excretion	
Series	No. animals in series	Milliunits post-pituitary ext. injected	Urine, cc/100 g B.W.	Chlorides, mg/cc urine
A	24	0	7.4	0.32
B	16	2.5	7.1	0.29
C	12	5	6.6	1.22
D	24	10	6.0	2.40
E	17	25	5.4	3.14
F	21	50	4.2	3.98
G*	6	375	2.7	6.05
H*	6	750	2.7	8.50
I*	12	1500	2.2	9.45
J*	6	3000	2.5	9.35

*Data taken from Table 3, Silvette, *Am. J. Physiol.*, 1940, **128**, 747.

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[1] Silvette, H., *Am. J. Physiol.*, 1940, **128**, 747.

or tolerance to the pituitary extract. The animals were given intraperitoneal injections of 10 cc of 0.2% sodium chloride solution per 100 g of body weight with which had been mixed the calculated amount of pituitary extract.* They were then placed in metabolism cages for 6 hours, and the urine collected and analyzed for chlorides.[1]

The data are presented in Table I. These show that as the dosage of the extract was progressively decreased, the excretion of water gradually increased while that of the chlorides steadily diminished. The water excretion approached the normal level much more rapidly than the chloride excretion, however; and, when the dosage of the extract was reduced to 5 milliunits, it was observed that the urine excretion was now inhibited to the extent of only 11%, while the chloride output was still 280% above the normal level. When the amount of pituitary extract was reduced still further (*e.g.*, to 2.5 milliunits) no significant change from the control levels could be observed. This dosage, therefore, was concluded to be subminimal, at least in respect to the chloride and water changes. Analogously, the dose of 3000 milliunits was supramaximal, as its effect on urinary excretion was no greater than that produced by an injection of 1500 milliunits.

Theoretically it should be possible to find an extract dosage between 5 and 2.5 milliunits at which the water excretion would be normal while the chloride excretion would still be above control levels, but experimentally this has not proved possible because the available extracts are not standardized with respect to their antidiuretic potency, and may indeed vary otherwise to the extent of ±20%.†

The above observations lend additional weight to the conception of the duality of the response to post-pituitary extract; *i.e.*, that the injection of the extract leads to 2 separate and distinct effects on kidney function, the one concerned with changes in salt excretion and the other with water.[1, 2] They also indicate that the chloride-concentrating ability of the renal tubules, which is enhanced by the antidiuretic hormone,[1] is considerably more sensitive to this hormone than is the parallel water-resorbing power. This difference in sensitivity might be useful as the basis for a practicable method of standardizing the antidiuretic potency of pituitary extract, since under the conditions of the experiments described herein the rate of change of the chloride excretion is considerably greater than that of the water output over a practicable working range of 5-25 milliunits of U.S.P. post-pituitary extract.

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* Posterior Pituitary Solution Squibb (U.S.P. XI strength), kindly furnished by Dr. John F. Anderson of E. R. Squibb and Sons.

† *United States Pharmacopoeia*, XI revision, 1936, article "Liquor Pituitarii Posterii."

[2] Melville, K. I., *J. Physiol.*, 1936, **87**, 129.

11765 P

l-Proline and Tumor Incidence in Mice.

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It has been demonstrated that l-proline is a specific participant in some process concerned in differentiation expression of obelia.[1]

It is our purpose and practice to test the repeatability or non-repeatability of the uncomplicated and unequivocal findings from lower forms on growth of spontaneous mammary tumors in female mice. Such has been done with l-proline.

Preliminary trials for toxicity level were made on some 20 test and 20 control mice by Mr. McDevitt at the Marine Experimental Station in North Truro. More secondary tumors appeared in the proline mice. These were separate new tumors. The small number of animals and the changing dosage made this observation inadequate for conclusion.

The measure of non-toxicity commonly used in these growth studies is absence of body weight loss of tests in terms of controls on prolonged administration of the amino acid.

Having established the safe dosage the regular series of trials was run in the mouse colony at Philadelphia. Here 78 mice were daily given by intrascapular subcutaneous injection 0.2 cc of an M/125 solution of l-proline in distilled water from the time of appearance of the first tumor until death. Seventy-six untreated mice served as controls. These lived, grew, produced their tumors and died contemporaneously with the others under like environmental and nutritive conditions.

Here, too, the mice given l-proline had more multiple primary tumors than the controls. All mice, both tests and controls, had primary tumors of course. A second tumor appeared in 56.4% of the proline group, and in but 44.7% of the controls. A third tumor appeared in 20.5% of the proline series and in but 13.2%

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[1] Hammett, F. S., and Collings, W. D., *Growth*, 1937, 285.

of the controls; and a fourth tumor came in 9.0% of the proline mice, and in but 2.6% of the controls.

Since we are here dealing with *more* instead of *larger* tumors it is well to distinguish between tumor production and tumor growth. Tumor production depends on the change from body cell to cancer cell. Tumor growth depends to a large degree on cell proliferation. It is conceivable that more tumors are produced, not because more cells are stimulated to divide; but because more body cells or nests of body cells are stimulated to undergo specialization into cancer cells.

If the argument is logically sound, it follows that these mouse tumor results are consistent with those obtained with obelia, since both may be interpreted as indicating a stimulation of cellular specialization or differentiation by l-proline.[2]

No less noteworthy is the fact that l-proline—a naturally occurring tissue component of general distribution—is perhaps a significant factor in tumor production.

Conclusion. Tumor incidence in mice is enhanced by l-proline, probably through its favoring action in some process concerned in cellular differentiation.

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Influence of Amorphous Fraction from Adrenal Cortex on Efficiency of Muscle.

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An extract of the adrenal cortex which is free from epinephrine and contains the physiologically active steroid derivatives characteristic of the adrenal cortex can be separated into a surprisingly large number of crystalline compounds and an amorphous fraction. The crystalline compounds A, B, E, and F, which have an atom of oxygen attached to C_{11}, have a marked effect on the efficiency of muscle[1] and on carbohydrate metabolism,[2, 3] but relatively large amounts are required to maintain renal function. When large amounts are administered to normal male rats these compounds produce atrophy of the adrenal and thymus glands.[4, 5] Desoxycorticosterone, which is identical with compound B except that there is no atom of oxygen on C_{11}, has but little effect on the efficiency of muscle and on carbohydrate metabolism, but it is from 6 to 10 times more efficient than compound B when renal function is used as a criterion. The amorphous fraction has the greatest effect on renal function and it protects the adrenalectomized animal against certain forms of stress such as a low environmental temperature, but even 40 times the amount of this fraction which is required to produce these effects does not cause atrophy of the thymus or adrenal glands. It has also been shown that the amorphous fraction has very little effect on carbohydrate metabolism.[6] It became desirable, therefore, to determine whether this fraction could increase the efficiency of muscle to a degree comparable to compounds A, B, E, and F.

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[2] Hammett, F. S., *The Nature of Growth,* Science Press and Printing Company, Lancaster, Pa., 1936.

[1] Ingle, D. J., *Endocrinology,* 1940, **26**. 472.

[2] Long, C. N. H., Katzin, B., and Fry, Edith G., *Endocrinology,* 1940, **26**, 309.

[3] Ingle, D. J., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 176.

In the present report it will be shown that very small amounts of the amorphous fraction will protect the adrenalectomized rat against a loss in body weight, but that animals so treated are incapable of sustained work. The compound 17-hydroxy-11-dehydrocorticosterone was more active in its effect on the efficiency of muscle than was the amorphous fraction.

Methods. Male rats of the Sprague-Dawley strain which weighed 180 ± 2.0 g were used. The diet was Purina Dog Chow. Bilateral adrenalectomy was performed in one stage under ether anesthesia. "Soap and water" cleanliness was maintained and no postoperative infections or other complications developed. The animals were either subjected to the work test immediately following the operation or they were maintained for one week before they were tested. The cortical hormones were dissolved in sesame oil and were administered by subcutaneous injection twice daily. The amount of sesame oil was kept constant at 1 cc per day for each rat. For the work test the animals were anesthetized with phenobarbital sodium. The left gastrocnemius muscle of each animal was weighted with 100 g and stimulated to contract 3 times per second. Each animal received 5 cc of water at the beginning of stimulation and 6 hours later. In Experiment 1, stimulation was continued until the rat ceased to respond or for a maximum of 24 hours. In Experiment 2, stimulation was continued until the muscle ceased to respond but

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[4] Wells, B. B., and Kendall, E. C., *Proc. Staff Meet., Mayo Clin.*, 1940, **15**, 133.

[5] Ingle, D. J., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 174.

[6] Wells, B. B., *Proc. Staff Meet., Mayo Clin.*, 1940, **15**, 294.

in every instance this was less than 24 hours. The details of the method have been described.[7, 8]

Experiments and Results. In Experiment 1, 42 adrenalectomized rats were used. Ten untreated rats were tested immediately following adrenalectomy. Eight rats were treated with the amorphous fraction, 12 rats with 17-hydroxy-11-dehydrocorticosterone and 12 rats with 17-hydroxy-11-dehydrocorticosterone acetate. The data on dosage and work performance are summarized in Table I. Five rats into which incisions had been made but without removal of the adrenal glands were subjected to the work test for 24 hours. The average amount of work performed by the latter rats was 21,651 recorder revolutions with a range of 17,199 to 25,026. The amorphous fraction increased the efficiency of the muscle above the level of the untreated rats less than did either 17-hydroxy-11-dehydrocorticosterone or its acetate. The acetate appeared to be less effective in this test than the nonesterified compound. A similar

TABLE I.
Work Performance of Rats Expressed as Recorder Revolutions Immediately Following Adrenalectomy.

	Work[3] performance under administration of			
Dose, mg*	Amorphous fraction	Compound E[1]	Compound E Acetate[1]	Work performance of untreated rats
0.125	5633[2]	10802[2]	4915[2]	6436[2]
	5047	3218	8583	6718
		11701	2377	2085
		11625	7924	2164
				4316
0.25	6377	12339	6230	3963
	8199	16124	10410	7702
		12483	12229	3003
		10374	12876	5153
				3618
0.50	9482	15133	11577	
	4036	13165	12590	
		14063	15731	
		13158	14177	
1.00	12953			
	15014			

*These amounts of the test substances were administered at the beginning of stimulation and again 6 hours later.

[1] 17-hydroxy-11-dehydrocorticosterone.

[2] Each recorder revolution is equivalent to approximately 400 gram-centimeters of work.

[3] All of the values for work below 9000 represent animals in which muscular responsiveness was lost before the completion of the 24-hour period. All of the values for work above 12000 represent animals which were continuing to work at the end of the 24-hour period.

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[7] Heron, W. T., Hales, W. M., and Ingle, D. J., *Am. J. Physiol.*, 1934, **110**, 357.

[8] Ingle, D. J., *Am. J. Physiol.*, 1936, **16**. 622.

relationship in the order of the activity of 11-desoxycorticosterone and its acetate in the 24-hour work test has been reported.[1]

In Experiment 2, 31 adrenalectomized rats were used; seven were untreated and 24 received varying amounts of the amorphous fraction for one week and were then subjected to the work test. The data on body weights and on work are summarized in Table II. Definite protection against a loss in body weight was afforded by as little as 5 μg per day of the amorphous fraction, but although the work performance was increased over that of untreated animals, it remained very poor as compared to the response of animals into which incisions had been made but without removal of the adrenal glands.

TABLE II.
Body Weight and Work Performance of Adrenalectomized Rats After Treatment with the Amorphous Fraction for One Week.

Dose, mg*	Weight, g[1]	Total work, recorder revolutions[2]
0	143	94
	145	185
	150	389
	142	151
	154	246
	146	579
	160	1034
.001	176	1446
	166	780
	151	435
	156	590
.002	175	2060
	160	824
	188	3661
	160	3087
.005	189	1243
	187	1657
	187	2592
	203	4884
.01	189	3040
	201	4907
	205	3269
	186	1446
.05	197	1513
	194	2566
	182	2948
	190	1993
.10	183	4748
	203	4335
	194	5930
	190	5139

*Total daily dose. Divided into 2 daily injections.

[1] The initial weight of each rat was 180 ± 2 g

[2] Each recorder-revolution is equivalent to approximately 400 gram-centimeters of work.

Rats so treated were found, in a recent study, to have values for total work of 87,645 to 131,261, with an average of 112,164 recorder revolutions.[9]

Comment. The amorphous fraction is highly effective in protecting the adrenalectomized rat against a loss of weight. It is the most effective fraction when tested with renal function as a criterion in the adrenalectomized dog. In these respects the compound 17-hydroxy-11-dehydrocorticosterone is ineffective. It does not protect the adrenalectomized rat against a loss in weight[9, 10] although in sufficient dose it will prolong its survival. Large amounts are required to prolong the life of the adrenalectomized dog.[10, 11] With respect to muscular work, however, the relationship is reversed. The compound 17-hydroxy-11-dehydrocorticosterone has a marked effect on the ability of the adrenalectomized rat to work immediately after operation and during its period of survival.[1, 9] The amorphous fraction given to the adrenalectomized rat either immediately after operation or during the period of survival appeared to be less effective when the maintenance of the efficiency of muscle was used as a criterion. The results of this and other studies[1–3, 6, 9] indicate that the compounds which have the greatest effect on carbohydrate metabolism also have the greatest effect on the efficiency of muscular activity and that the compounds that have the greatest effect on renal function have a relatively slight effect on carbohydrate metabolism and on the efficiency of muscle.

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Ineffectiveness of Histaminase in Anaphylactic Shock in Guinea Pigs.*

ALFRED F. KNOLL. (Introduced by R. R. Mellon.)

*From the Western Pennsylvania Hospital Institute of Pathology, Pittsburgh, Pa.**

Best and McHenry's[1,2] discovery of a histamine-inactivating substance in the gastro-intestinal tract and kidney of the dog initiated various observations upon the effect of this material in certain well established histamine reactions. Certain workers were unable to produce any change of the histamine effect upon the guinea pig uterus,[3] the blood pressure of cats and rabbits,[3] the bronchial volume of cats,[3] or the gastric response in humans,[3, 4] and dogs.[5] With oral, intravenous, or subcutaneous use of histaminase, Bosse[6] failed to demonstrate any effect upon the Shwartzman phenomenon in rabbits.

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[9] Ingle, D. J., *Endocrinology*, 1940, **27**, 297.

[10] Wells, B. B., and Kendall, E. C., *Proc. Staff Meet., Mayo Clin.*, 1940, **15**, 324.

[11] Ingle, D. J., and Mason, H. L., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 154.

* The author gratefully acknowledges the donation of histaminase by the Winthrop Chemical Company, New York, N. Y., and by Sharp and Dohme, Philadelphia, Pa.

[1] Best, C. H., *J. Physiol.*, 1929, **67**, 256.

[2] Best, C. H., and McHenry, C. W., *J. Physiol.*, 1930, **70**, 349.

However, other investigators report a rather striking histamine-inactivation with histaminase. Included in these reports is the work of MacGregor and Peat,[7] Suzuki,[8] Unger and Bolgert.[9] Of special interest is the report of Karady and Browne[10] who were able to prevent death in all of 20 guinea pigs subjected to anaphylactic shock by pretreatment of these animals with histaminase injected into the jugular vein.

The following experiments were performed in an attempt to confirm the results of Karady and Browne.[10]

Experiment I. Fifteen guinea pigs averaging 350 g in weight were sensitized by the intraäbdominal injection of a 1 :4 egg albumin-normal saline mixture. Fourteen days later 7 of the animals were given one unit of histaminase (Sharp and Dohme TDS-638) in saline by the intraäbdominal route and about 5 hours later all animals received a shocking dose of one cc of 1 :2 egg albumin-normal saline mixture intraäbdominally. The intracardiac method of injection,

TABLE I.

	Controls			Histaminase		
Exp.	No.	Dead	Survivals	No.	Dead	Survivals
I*	7	5	2	6	0	6
II†	8	4	4	9	4	5
III†	11	7	4	12	6	6
IV*	17	5	12	17	6	11
V†	32	20	12	32	19	13
Total	75	41	34	76	35	41

*Histaminase of Sharp and Dohme Co., Philadelphia, Pa.
†Histaminase of Winthrop Chemical Co., New York, N. Y.

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[3] Felix, I., *Acta Med. Scandinav.*, 1938, **95**, 1.

[4] Biguria, F., and Canzanelli, A., *Am. J. Physiol.*, 1934, **110**, 243.

[5] Atkinson, A. J., and Ivy, A. C., *Am. J. Physiol.*, 1934, **107**, 168.

[6] Bosse, M. D., *J. Lab. and Clin. Med.*, in press.

[7] McGregor, R. G., and Peat, S., *J. Physiol.*, 1933, **77**, 310.

[8] Suzuki, R., *J. Med. Assn. Formosa, Taiwan, Igakkai Zasshi*, 1937 **47**, 68.

[9] Ungar, G., and Bolgert, M., *Compt. rend. soc. d. Biol.*, 1938, **192**, 1107.

[10] Karady, S., and Browne, J. S. L., *J. Immunol.*, 1939, **37**, 463.

used in two animals, was discarded because death resulted within 30 seconds. The results are tabulated in Table I.

Autopsy disclosed pulmonary edema and emphysema in all of the animals that died.

Experiment II. Seventeen guinea pigs averaging 300 g were sensitized with one cc of 1:1 egg albumin-saline mixture intraäbdominally. Fourteen days later 9 animals were given one unit of histaminase (Winthrop T-360) in saline intraäbdominally. All animals received a shocking dose of one cc of 1:1 egg albumin-normal saline mixture by the same route 4 hours later (Table I). Autopsy revealed pulmonary edema and emphysema in only 2 animals of each group of 4 that died.

Experiment III. Twenty-four guinea pigs averaging 300 g were sensitized by intraäbdominal injection of 1:1 egg albumin-normal saline mixture. Fourteen days later 12 animals received a subcutaneous injection of 2 cc of a freshly prepared saline suspension containing 3 units of histaminase (Winthrop T-360). At the same time 5 control animals were given a shocking dose of 0.5 cc of 1:1 egg albumin-saline mixture. In 24 hours a second similar subcutaneous injection of histaminase was administered to the first 12 animals. Four hours later all animals were given an intraäbdominal shocking dose of 0.5 cc of 1:1 egg albumin-normal saline mixture intraäbdominally. The results are given in Table I. In the control group one animal was excluded because of accidental death. In the histaminase group one death occurred without the findings of anaphylactic shock at autopsy. Autopsy of all other fatalities disclosed the typical pulmonary edema and emphysema.

Experiment IV. Thirty-four guinea pigs averaging 250 g were sensitized by the intraäbdominal injection of one cc of 1:1 egg albumin-normal saline mixture. Fourteen days later, 17 animals received one unit of histaminase (Sharp and Dohme 777-L5) intra-abdominally. The shocking dose of 0.5 cc of 1:1 egg albumin-normal saline mixture was administered to all animals 4 hours afterwards. The results are tabulated in Table I. Autopsy showed characteristic pulmonary edema and emphysema in all animals that died, except one in the histaminase group.

Experiment V. In this group the technic of Karady and Browne[10] was employed. Sixty-nine guinea pigs were sensitized with 3 cc of a 1:1 egg albumin-normal saline mixture subcutaneously. On the 16th day, 32 of the animals received 3 units of histaminase (Winthrop T 360) into the jugular vein (without anesthesia) 15 minutes before an intraäbdominal shocking dose of 2 cc of 1:1 egg albumin-normal saline mixture, while 5 were given the histaminase into the jugular vein without the subsequent shocking dose of egg albumin. The latter 5 animals showed no ill effect of the intravenous injection. The results are given in Table I. Autopsy of the fatalities disclosed typical pulmonary edema and emphysema in 17 of the controls, while in 2 animals the emphysema was patchy, and in one animal gross lesions were absent. In the fatalities of the histaminase group, findings typical of anaphylaxis were present in 18 of the animals, while in the remaining one, emphysematous blebs were present.

Discussion. The first experiment involving a small group in which the administration of histamine apparently effected a survival rate of 100%, was very promising and appeared to confirm the findings of Karady and Browne. However, subsequent experiments involving a total number of animals more than 3½ times as great as that used by the above workers failed to show any effect of the histaminase preparations on anaphylactic shock of guinea pigs. The results were negative when the histaminase preparations were injected intravenously or intraäbdominally.

Conclusion. The protective action of histaminase on anaphylactic shock of guinea pigs as reported by Karady and Browne could not be confirmed.

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Bacteriostatic and Bactericidal Substances Produced by a Soil Actinomyces.*

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The ability to antagonize other microörganisms is widely distributed among actinomycetes, a large group of microörganisms, present in soils, composts, water basins, dust, and in other natural substrates.[1] The nature of the antagonistic action exerted by these organisms is not always the same: either fungi or bacteria were reported to be affected, dead or living cells of bacteria, as well as various types of bacteria. These varying observations point to the fact that one is dealing not with a single type of reaction in all cases, but with different mechanisms, possibly characteristic of the different antagonistic organisms.

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* Journal Series Paper, New Jersey Agricultural Experiment Station, Department of Soil Chemistry and Microbiology.

[1] Greig-Smith, R., *Proc. Linn. Soc. N. S. Wales*, 1917, **42**, 162; Lieske, R., *Morphologie und Biologie der Strahlenpilze*, Leipzig, 1921, p. 138; Gratia, A., and Dath, S., *Compt. rend. Soc. Biol.*, 1925, **93**, 451; 1926, **94**, 1267; Welsch, M., *Compt. rend. Soc. Biol.*, 1937, **124**, 575; Millard, W. A., and Taylor, C. B., *Ann. Appl. Biol.*, 1927, **14**, 202; Borodulina, J. A., *Microbiologia*, 1935, **4**, 561; Waksman, S. A., and Foster, J. W., *Soil Sci.*, 1937, **43**, 69; Nakhimovskaia, M. I., *Microbiologia*, 1937, **6**, 131; Krassilnikov, N. A., and Koreniako, A. I., *Ibid.*, 1939, **8**, 673.

By a method previously described,[2] the authors isolated from the soil a species of *Actinomyces* which has marked bacteriostatic and bactericidal effects upon all bacteria, belonging both to the Gram-positive and Gram-negative types. This organism was found to belong to the chromogenic type of actinomycetes, characterized by the production of black pigments on protein- and peptone-containing media. No relation was found between the formation of this pigment and the active substance as will be shown in detail elsewhere,[3] since the substance is also formed on protein-free synthetic media, though in lower concentrations. Certain amino acids, such as phenyl-alanine favor its formation; however, it is produced in media containing sodium nitrate and starch as the only sources of nitrogen and carbon.

The organism was grown on agar media, for 6-8 days, and the active substance extracted with ether; it was redissolved in 95% alcohol, to give a stock solution containing 5 mg per 1 cc; for making the tests, the alcoholic solution was diluted with water. The aqueous solution was turbid; however, when the ether-soluble material was treated with petrol ether, a readily crystallizable preparation was obtained which gave a clear aqueous solution. The active substance could thus be separated into 2 fractions: (A) soluble in ether and in alcohol, but not in petrol ether, and giving a clear solution in water; (B) soluble in ether and in petrol ether, soluble with difficulty in alcohol, and giving a turbid suspension with water. Fraction A was bright red in color and gave a yellow solution even in concentrations of 0.001 mg per 1 cc; it possessed extremely high bacteriostatic properties, but was rather slowly bactericidal. Fraction B was colorless, had comparatively little bacteriostatic action and was markedly bactericidal. It may be added here that the younger cultures of the organism had a higher concentration of the B fraction, as compared with the A fraction; as the culture grew older, the relative yield of the former diminished and of the latter increased. The active substance was tentatively designated as "actinomycin". It is proposed here to designate the above two preparations as "actinomycin A" and "actinomycin B".

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[2] Waksman, S. A., and Woodruff, H. B., *J. Bact.*, 1940, **40**, 581.

[3] Waksman, S. A., and Woodruff, H. B., *Soil Sci.*, 1940, **50**.

A comparative study of the inhibitory effect of the active substance against a large number of bacteria, using solid or liquid culture methods, brought out the fact that many of the Gram-positive bacteria were largely or completely inhibited by a concentration of 0.1 mg per liter of medium; some were inhibited even by a concentration of 0.01 mg of purified actinomycin A added per liter of nutrient agar or broth; other Gram-positive bacteria required 10 mg per liter for complete inhibition. The 2 species of *Actinomyces* tested were found to be partly or largely inhibited by 10 mg per liter. The Gram-negative bacteria also varied considerably in this respect; some were inhibited even by 100 mg per liter of medium, whereas the growth of others was markedly reduced by 10 mg per liter; the colon-aerogenes and *Serratia* groups were the most resistant (Table I).

In testing the bactericidal properties of the 2 preparations, various

TABLE I.
Inhibitory Effect of Actinomycin upon the Growth of a Number of Bacteria.

Organism	Gram stain	Actinomycin added per liter of medium, mg			
		0.1	1.0	10	100
Serratia marcescens	—	3[1]	3[1]	3[1]	3[1]
Aerobacter aerogenes	—	3	3	3	3[2]
Escherichia coli—intermediate	—	3	3	3	3[2]
Escherichia coli	—	3	3	3	1[2]
Pseudomonas aeruginosa	—	3	3	3	0
Pseudomonas fluorescens	—	3	3	3	0
Brucella abortus	—	3	3	3	0
Neisseria catarrhalis	—	3	3	2	0
Erwinia carotovora	—	3	3	2	0
Shigella gallinarum	—	3	2	2	0
Achromobacter stutzeri	—	3	2	1	0
Hemophilus pertussis	—	3	3	0	0
Azotobacter vinelandii	—	3	0	0	0
Actinomyces cellulosæ	+	3	2	1	0
Actinomyces californicus	+	3	3	2	0
Mycobacterium tuberculosis	+	3	3	0	0
Clostridium welchii	+	3	0	0	0
Bac. macerans	+	3	3	0	0
Bac. megatherium	+	3	0	0	0
Bac. polymyxa	+	3	0	0	0
Bac. mycoides	+	1	0	0	0
Bac. mesentericus	+	1	0	0	0
Bac. cereus	+	1	0	0	0
Bac. subtilis I	+	0	0	0	0
Bac. subtilis II	+	0	0	0	0
Gaffkya tetragena	+	0	0	0	0
Sarcina lutea	+	0	0	0	0
Streptococci and Staphylococci	+	0	0	0	0

[1] 0—No growth; 1—Trace; 2—Fair growth; 3—Good growth.

[2] 200 mg per liter usually gave for *A. aerogenes* — 2, *E. coli* int. — 1, and *E. coli* — 0.

methods were employed for different organisms. *Brucella abortus* and *Sarcina lutea* were used as washed suspensions; *Escherichia coli* was grown in broth cultures, for 5 hours at 37°, and the 2 preparations added. *Bacillus subtilis* was grown in broth cultures and 0.2 cc portions added to 100 cc of a phosphate buffer solution and incubated at 28°C.

Actinomycin A exerted, in small amounts, a definite bacteriostatic effect upon the growth of *Bac. subtilis* (Table II), but no bactericidal action; this is clearly brought out by the lack of cell multiplication with 0.01 and 0.1 mg of the substance. In larger concentrations, especially after longer periods of incubation, the A fraction exerted a definite bactericidal effect. On the other hand, actinomycin B had no growth-inhibiting effect in small doses, as shown by the multiplication of the organism with the lower concentrations of the substance; in larger concentrations, it had a marked bactericidal action. The same effects were obtained (Table III) for the 2 preparations acting upon *B. abortus*.

A study of the mechanism of the bactericidal action of actino-

TABLE II.

Bactericidal Action of Actinomycin Fractions upon *Bacillus subtilis*.
Cells added to 100 cc buffer solution—580,000; spores—217,000.

Preparation used	Mg added to 100 cc buffer	No. of viable cells left		
		After 24 hr	After 72 hr	
		Total cells	Total cells	Spores
0	0	2,490,000	15,000,000	61,000
Actinomycin A	0.01	470,000	200,000	32,000
" A	0.10	510,000	150,000	5,000
" A	1.00	180,000	130,000	<1,000
" B	0.01	2,760,000	46,800,000	18,000
" B	0.10	1,040,000	43,600,000	<1,000
" B	1.00	130,000	180,000	17,000

TABLE III.

Bactericidal Action of Actinomycin Fractions upon *Brucella abortus*.
2,530,000,000 per 100 cc of buffer solution.

Preparation used	Mg added to 100 cc buffer	No. of viable cells left	
		After 20 hr total cells	After 72 hr total cells
0	0	4,260,000,000	427,000,000
Actinomycin A	0.1	2,690,000,000	8,000,000
" A	2.0	1,290,000,000	<100,000
" A	5.0	221,000,000	<100,000
" B	0.1	3,990,000,000	210,000,000
" B	2.0	<100,000	<100,000
" B	5.0	<100,000	<100,000

TABLE IV.
Destruction of *E. coli* by Varying Concentrations of Actinomycin.

Actinomycin mg	No. of surviving *E. coli* cells, as determined by plate method, after			
	3 hr	7 hr	24 hr	48 hr
0	215,600,000	231,200,000		238,000,000
0.01	224,000,000	264,200,000	232,800,000	154,400,000
0.10	250,800,000	196,200,000	91,200,000	21,600,000
1.00	126,800,000	44,100,000	0	0

mycin, as measured by the viability of the bacterial cells on culture media and by the methylene blue reduction test brought out the fact that in a 10 cc suspension of *E. coli*, 0.5 mg actinomycin reduced the numbers of living cells from 6,400,000 to 493,000, the M.B. test remaining —: 1 mg of the substance reduced the number of cells to 4,800 with M.B. —: 2 mg brought about complete destruction of the total number of cells.

The destruction of the bacterial cells by actinomycin seems to be due to a chemical interaction, similar to that of other antiseptics. This can be shown by analyzing the results obtained with 0.1 mg actinomycin added to a suspension of *E. coli* cells in a 10 cc buffer solution (Table IV).

$$K = \frac{1}{\text{time}} \log \frac{\text{No. of cells at beginning}}{\text{No. of cells at time—t}}$$

$$7\text{ hr} \quad K = 1/4 \ \log \frac{250}{196} \qquad K = 0.026$$

$$24\text{ hr} \quad K = 1/21 \log \frac{250}{91} \qquad K = 0.021$$

$$48\text{ hr} \quad K = 1/45 \log \frac{250}{22} \qquad K = 0.023$$

Summary. The bacteriostatic and bactericidal substance produced by a soil *Actinomyces* was shown to consist of 2 compounds, designated as "actinomycin A" and "actinomycin B". The first is highly pigmented (red) and is soluble in ether, ethyl alcohol and water, but not in petrol ether: it gives a clear solution in water. The second is soluble in ether and in petrol ether, soluble with difficulty in alcohol, but not in water. The first (A) is highly bacteriostatic, many Gram-positive bacteria being inhibited by dilutions of 1:100,000,000; the Gram-negative bacteria are inhibited only by higher concentrations, namely, 1:5,000 to 1:100,000; this substance is only weakly bactericidal. The second substance (B) is weakly bacteriostatic, but more actively bactericidal. Although Gram-negative bacteria are as a rule more resistant against the actinomycin than the Gram-positive forms, there is no sharp line of demarcation between the two groups. Marked differences in sensitivity exist between the bacteria within each group.

11769

Germicidal Efficiency of Some Medicinal Dyes Compared to a Group of Non-Dye Disinfectants.

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The phenol coefficient test is employed universally for rating disinfectants. The test was designed originally for the evaluation of germicides which were closely related to phenol in structure. However, the test is of little value for determining the efficiency of compounds which are unlike phenol. Also, the test gives no information concerning the degree of inactivation of a germicide in the presence of organic matter, nor its toxicity towards living tissue.

In a series of papers published over a number of years[1] a new method was proposed and employed for the evaluation of germicidal substances intended for clinical application. The germicides were tested for their effect on living embryonic chick heart tissue cultivated *in vitro* as well as for their ability to kill bacteria. All tests were performed at 37°C and in the presence of a standard amount of organic matter. A number known as the toxicity index was determined which was defined as the ratio of the highest dilution of germicide required to kill the tissue in 10 minutes to the highest dilution required to kill the test organism in the same period of time under identical conditions. The smaller the toxicity index the more nearly perfect the chemotherapeutic agent. Theoretically, an index less than one means that the germicide is more toxic to bacteria than to the embryonic tissue while an index greater than one means that the germicide is more toxic to the embryonic tissue than to the bacteria.

Some of the dyes, especially those belonging to the triphenylmethane group, are used clinically and strong claims have been made for their efficiency. At one time they were employed to a considerable extent but they have been largely replaced by other germicidal agents.

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[1] Salle, A. J., Shechmeister, I. L., and McOmie, W. A., *J. Bact.*, 1939, **37**, 639.

Some of the characteristics of dyes are: (1) They are strongly bacteriostatic. This property, more than their germicidal action, probably explains the favorable results obtained from their use; (2) they are markedly inactivated in the presence of organic matter. This property is more pronounced in the case of the dyes than in the non-dye compounds; (3) they are relatively unstable in solution and especially in the presence of light; (4) the triphenylmethane dyes and probably others are more effective against Gram-positive than Gram-negative bacteria.

A number of medicinal dyes were rated by this method[1] and the toxicity indices determined. The organisms used were *Staphylococcus aureus* and *Eberthella typhosa.* The results are given in Table I. It is seen that with the exception of brilliant green the dyes tested were unable to kill *E. typhosa* in 10 minutes at 37°C, in the presence of a standard amount of organic matter. The efficiency of brilliant green is very poor, the index showing that it is 161 times more toxic to tissue than to *E. typhosa* under the conditions of the test. However, the dyes rated better against *Staphylococcus aureus* (Gram positive) than against *E. typhosa* (Gram negative). All of the triphenylmethane dyes killed *Staph. aureus* and the toxicity indices were much lower in every case. It may be seen that there is no relationship between toxicity indices and phenol coefficients.

TABLE I.
Toxicity of Dyes to Chick Heart Tissue and Bacteria.

Compound	Killing dilution		Toxicity Index = A/B	Phenol Coefficient
	Tissue = A	Bacteria = B		
	Eberthella typhosa.			
Brilliant green	33,600	208	161	1.1
Crystal violet	60,800	will not kill	....	....
Methyl ” B	25,000	” ” ”	....	....
” ” 6B	25,000	” ” ”	....	....
” ”	25,000	” ” ”	....	....
Methylene blue	7,000	” ” ”	....	....
Proflavine	3,200	” ” ”	....	....
Acriflavine base	3,000	” ” ”	....	....
” HCl	2,700	” ” ”	....	....
	Staphylococcus aureus.			
Brilliant green	33,600	5,500	6.0	50.0
Crystal violet	60,800	2,800	21.8	25.5
Methyl ” B	25,000	1,200	20.8	10.9
” ” 6B	25,000	1,150	21.4	10.5
” ”	25,000	1,150	21.4	10.5
Methylene blue	7,000	will not kill	....	....
Proflavine	3,200	” ” ”	....	....
Acriflavine base	3,000	” ” ”	....	....
” HCl	2,700	” ” ”	....	....

TABLE II.
Toxicity of Germicides to Chick Heart Tissue and Bacteria.

	Eberthella typhosa Killing dilution					*Staphylococcus aureus* Killing dilution			
Compound	Tissue A	Bacteria B	Toxicity Index A/B	Phenol Coefficient	Compound	Tissue A	Bacteria B	Toxicity Index A/B	Phenol Coefficient
Halogens:					Halogens:				
Azochloramid	7,800	101,000	0.08	543	Iodine	650	3,520	0.2	32
Iodine	650	3,370	0.20	18	Azochloramid	7,800	26,000	0.3	236
Chloramine	900	2,000	0.45	11	Chloramine	900	2,000	0.45	18
Silver compounds:					Phenols:				
Silver lactate	100	690	0.15	3.7	Hexylresorcinol	1,450	1,620	0.9	15
,, citrate	610	4,500	0.14	24	P-hydroxy phenyl-				
,, nitrate	140	1,250	0.11	6.7	n-amyl sulfide	2,800	2,700	1.0	25
,, protein					O-n-hexylphenol	3,125	2,900	1.1	26
strong	25	175	0.14	1.0	P-hydroxy-di-				
Silver protein					phenyl sulfide	3,125	2,500	1.3	23
mild	30	70	0.43	0.4	Phenol	224	110	2.0	—
Phenols:					Silver compounds:				
Hexylresorcinol	1,450	1,770	0.8	9.5	Silver lactate	100	110	0.9	1.0
P-hydroxy phenyl-					,, citrate	610	600	1.0	5.5
n-amyl sulfide	2,800	2,700	1.0	14.5	,, nitrate	140	80	1.8	0.7
O-n-hexylphenol	3,125	2,800	1.1	15.0	,, protein				
P-hydroxy diphenyl					strong	25	15	1.7	0.14
sulfide	3,125	3,125	1.0	17.0	Silver protein				
Phenol	224	186	1.2	—	mild	30	12*	2.5+	0.11—
Mercurials:					Mercurials:				
Metaphen	2,070	5,660	0.4	30.5	Metaphen	2,070	1,370	1.5	13
Mercurochrome	90	140	0.6	0.8	Mercurochrome	90	12½*	7.2+	—
Merthiolate	4,220	2,660	1.6	14.0	Merthiolate	4,220	25*	169.0+	—

*Failed to kill. A more concentrated solution could not be prepared.

For purposes of comparison results of tests on other germicides are included (Table II). It may be seen that the dyes rate very poor in comparison to some well-known germicides of a non-dye nature.

Summary. The dyes as a group are extremely poor germicidal agents. Only the triphenyl methane dyes were capable of killing *E. typhosa* and *Staph. aureus.* Brilliant green was the only dye that killed *E. typhosa* while several killed *Staph. aureus* but their extremely high toxicity to tissue showed that they are inferior to the better known non-dye germicides. Further work on the dyes is now in progress.

11770

The Relation of the Nervous System to Skin Potentials in the Intact Frog.

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Considerable has been written about the relation of nerve stimulation to the action potential across the frog's skin; but, to our knowledge, little is known concerning the influence of the brain and spinal cord on the resting potential of the intact animal. Roeber[1] was the first to note a definite negative variation across the frog's skin following nerve stimulation. Later Engelmann,[2] Hermann[3] and Bayliss and Bradford[4] showed an association between the degree of response to nerve stimulation and the secretory activity of the gland cells of the skin, as well as seasonal variations. Furthermore, Orbili[5] investigated in a similar way the influence of various concentrations of salts. Although these observations are of significance, it should be pointed out that they were performed either on the isolated nerve skin or after the frogs were pithed or curarized.

Since our observations show that injury to the brain and spinal cord causes definite changes in the resting potential across the skin in the intact animal we feel justified in reporting our results. Although Francis and Pumphrey[6] report no pronounced difference between the amount of potential across the intact and isolated skin our observations show that the nature of the resting potential reacts very differently in the two. The experimental procedure used was to fasten the frog, back down, on a frog board and to make a small incision in the ventral skin surface, just anterior to the cloaca. Through this opening one end of an agar-chloride bridge was inserted just under the skin, almost up to the xyphoid process. The end of the bridge was bent nearly at right angles and formed a cup which made a snugly fitting contact with the inside of the skin, the approximate area of its end being 25 sq mm. In no instance did any undue stretching of the skin occur. The other end of the bridge was led into a container of Ringer's solution into which a calomel electrode was inserted and connected with a potentiometer. In a similar manner a second agar-chloride bridge, of similar size and dimensions, was adjusted to the outside of the skin so as to fit directly over the inside bridge. A few drops of Ringer's solution were applied between the skin and the outside bridge to assure a good contact.

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[1] Roeber, H., *Arch. f. Anat. u. Physiol.,* 1869, 633.

[2] Engelmann, W., *Pfluger's Arch.,* 1872, **6**, 97.

[3] Hermann, L., *Pfluger's Arch.,* 1878, **17**, 291.

[4] Bayliss, W. H., and Bradford, J. R., *J. Physiol.,* 1886, **7**, 217.

[5] Orbili, A. L., *Z. f. Biol.,* 1910, **54**, 329.

[6] Francis, W. L., and Pumphrey, R. J., *J. Exp. Biol.,* 1933, **10**, 379.

With this technic the resting or basal potential remained remarkably steady, reaching a constant state within 5 to 10 minutes after the bridges were first adjusted. In no case was a drift recorded which lasted 30 or more minutes, as usually found for the isolated skin. Removing or changing the position of the electrodes had very little effect on the potential readings.

The effects of the central nervous system on this preparation were investigated as follows: After a control or constant E.M.F. was obtained, the bridges were removed and the frog was taken from the board long enough to destroy the brain (single pith). Following the pithing, the frog and the bridge electrodes were replaced in a position conforming as closely as possible to that in which the control readings had been taken. The time involved to carry out this procedure was approximately 2 to 3 minutes.

Within 3 minutes after the animal and electrodes were in position, potential readings were again taken for 4 consecutive 3-minute periods. Then, by similar manipulation, the spinal cord was destroyed (double pith), and the potential readings were recorded for another 12-minute period.

Result. In the presentation of the data (Table I) the millivolts obtained on the various frogs before pithing are recorded, but any variation from the normal or constant state in skin potential, following pithing, is tabulated as a percentage of the original potential.

It was surprising that a sudden and consistent fall of potential invariably followed both single and double pithing. Although the fall

TABLE I.
Skin Potential in Intact Frog, Following Single and Double Pithing.

No. of Exp.	I Millivolts at beginning of Exp.	II Single pithing (Time in min.) 3	6	9	12	III Double pithing (Time in min.) 3	6	9	12
Frog I	111	72	86	85	74	35	36	35	34
” II	41	54	61	66	66	46	49	49	49
” III	77	84	70	69	69	52	49	49	48
” IV	52	98	92	82	84	61	58	58	56
” V	33	76	73	70	70	51	51	48	45
” VI	40	88	85	83	83	65	65	68	68
” VII	80	88	78	75	75	62	62	60	60
” VIII	46	70	68	68	68	46	46	42	42
” IX	35	66	66	69	69	49	49	48	46
” X	68	75	75	75	75	35	35	34	34
	Avg	77	75	74	73	50	49	49	48

Column I represents actual skin potential in millivolts at the beginning of the experiment.

Column II represents the percentage of the original potential following single pithing. Readings were taken at 3-minute intervals for a period of 12 minutes.

Column III represents the additional percentage change in potential following double pithing. Readings were taken at 3-minute intervals for a period of 12 minutes.

in potential varied in different frogs, the average fell to about 75% following single pithing, and approximately 50% after double pithing, a decrease of approximately 25% in each case.

In view of these observations, it occurred to us that if the nervous system was related to skin potential, strychnine should augment

TABLE II.
Skin Potential in Intact Frog Following Injection of Strychnine

No. of Exp.	I Millivolts at beginning of Exp.	II Time in min. 3	6	9	12	15	18	21	24
Frog I	47	138	138	157	*178	144	108	87	83
” II	57	107	106	112	* 77	79	81		
” III	52	106	114	125	123	116	104	98	98
” IV	43	126	111	107	107	105	100	93	93
” V	49	127	108	105	103	* 83	108	108	103
” VI	66	133	133	114	* 88	86	80	80	
” VII	58	117	124	110	103		88	88	80
” VIII	62	129	132	116	112	101	97	89	84
” IX	30	140	126	123	116	116	113	110	103
” X	52	125	119	111	105	105	96	89	81
	Avg	125	121	118	111	104	98	93	91

Column I represents actual skin potential in millivolts at the beginning of the experiment.

Column II represents the percentage of the original potential following intraperitoneal injection of strychnine sulphate (0.25 cc of 1 in 1000). Readings were taken at 3-minute intervals for a period of 24 minutes in most cases. Asterisk indicates onset of convulsions.

TABLE III.

Skin Potential in Intact Frog Following Impairment of Nervous and Circulatory Systems.

	I	II				III			
		Before removing heart				After removing heart			
	Millivolts at beginning	(Time intervals in min.)							
No. of Exp.	of Exp.	3	6	9	12	3	6	9	12
Frog I	35	60	60	60	60	60	60	60	60
,, II	28	79	84	84	82	79	79	79	79
,, III	41	73	73	72	73	76	76	78	79
,, IV	60	75	68	68	68	67	67	67	67
,, V	45	57	56	56	56	56	57	56	56
	Avg	69	68	68	68	68	68	68	68

Column I represents actual skin potentials in millivolts at the beginning of the experiment.

Column II represents the percentage of the original potential following double pithing. Readings were taken at 3-minute intervals for a period of 12 minutes.

Column III represents the percentage of the original potential following double pithing and impairment of the circulatory system by removing the heart. Readings were taken at 3-minute intervals for a period of 12 minutes.

it. To test this, potentials of 10 normal frogs were recorded and then 0.25 cc of strychnine sulphate 1 in 1000 was injected intraperitoneally. All 10 frogs showed an increase in E.M.F. above the equilibrium potential (6 to 40%) within 3 minutes after the injection, and continued to show a positive potential for 12 to 15 minutes (Table II). Twenty-four minutes after the injection the potential had returned to normal or slightly below. Although the dosage was estimated to be below the convulsive level, it was interesting to note that in 3 of the 4 cases in which convulsions did occur, a sudden drop in potential followed.

As to the mechanism involved in producing these sudden changes in potential, nothing can be said at the present time. However, the results obtained after destruction of the nervous system or administration of strychnine indicate that the skin potential in intact animals is under a type of tonic control from the central nervous system. This is also borne out by a limited number of experiments in which the skin was denervated by sectioning all possible nerve connections to the skin surface. The result was a fall of skin potential of approximately the same magnitude as that produced by pithing the animal.*

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* Although these experiments show that the destruction of the nervous system causes a lowering of potential, it should be pointed out that if a frog with an opening in its ventral skin surface is put back into water for some time, there is a tendency to produce an increase in skin potential after brain and spinal cord destruction. What the cause of this change is, we do not know, but it may be related to disturbances in the isotonicity of the inside surface of the skin.

Since skin potential seems to be in some ways related to the nervous system, the question arose whether there is also a relation between skin potential and the circulatory system. A number of frogs were prepared as in previous experiments and, after equilibrium potentials had been reached, the frogs were double pithed; then, after readings had been taken for 12 minutes, the heart was removed or injured through a small opening. As the data indicate (Table III), there was no appreciable change in potential for a 12-minute period after the operation as compared with the immediate change following injury of the nervous system. This steady state following removal of the heart was maintained for 30 to 40 minutes, after which there was a gradual but definite fall in skin potential.

Conclusions. I. Potentials across the skin in the intact frog remain remarkably constant. Manipulation of electrodes or position of the frog has little influence on potential readings. II. Destruction of the nervous system causes a sudden decrease in skin potential. The average decrease in potential after brain and spinal cord destruction is approximately 25% in each case. III. Following intraperitoneal injection of strychnine sulphate into the intact frog there is an increase in skin potential for approximately 15 minutes, after which there is a gradual return to normal. IV. Alterations in circulation by removal of the heart causes no appreciable change in skin potential.

11771

Pantothenic Acid and Hemorrhagic Adrenal Necrosis in Rats.

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A hemorrhagic necrosis of the adrenal cortex was frequently observed in rats used for a study of vitamin B_6 deficiency in this laboratory. This abnormality was prevented when Vitab or a filtrate factor concentrate was included in the diet. Similar observations were reported by Daft and Sebrell.[1] When synthetic pantothenic acid became available it seemed desirable to investigate its relation to this adrenal pathology. While these investigations were being completed, Daft, Sebrell, Babcock, and Jukes[2] reported similar studies. The data herein presented are essentially a confirmation of the results obtained by the above workers.

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[1] Daft, F. S., and Sebrell, W. H., *Public Health Rep.*, 1939, **54**, 2247.

[2] Daft, F. S., Sebrell, W. H., Babcock, S. H., Jr., and Jukes, T. H., *Public Health Rep.*, 1940, **55**, 1333.

The basal diet used in these studies consisted of alcohol-extracted Labco casein 18, sucrose 78, and salts No. 186[3] 4%. This diet was supplemented in all experiments with adequate amounts of thiamin, riboflavin, choline, calciferol, and carotene. Pyridoxine was given in some cases from the start of the experiment and in others after severe skin lesions had developed. In one experiment butterfat was included in the basal diet at 5, 10, or 20% levels without affecting the incidence of adrenal necrosis. Other dietary supplements are listed in Table I.

Severe adrenal cortical hemorrhage and necrosis was not produced in every case where pantothenic acid was omitted from the diet. However, it was never present in the rats which received a source of natural pantothenic acid or synthetic calcium pantothenate.

The animals which presented severe necrotic changes in the adrenal cortex failed rapidly, developed a severe hemorrhagic rhinitis, and, in some cases, passed into coma before they were removed for examination. Microscopic examination was made of necropsy material consisting of sections of adrenal gland, heart, spleen, liver, kidney, thymus, lung, and the intraorbital lacrimal gland. The adrenal hemorrhage originated in the inner zone of the cortex, but the medulla was not involved. Abnormal thymic involution was present in the animals with adrenal necrosis. Pneumonic processes were present in the lungs in most of these animals. They were, however, more severe and sometimes involved extensive consolidation of the lung tissue in the filtrate-factor-deficient animals. The intraorbital lacrimal glands from the filtrate-factor-deficient animals showed mild epithelial sloughing and some hemorrhage into

TABLE I.

		No. of cases and type of lesion	
Daily dietary supplements	No. of rats	Severe hemorrhagic adrenal necrosis	Adrenal hemorrhage only
300 mg Vitab*	10	none	none
Filtrate factor 2†	7	”	”
100 mg Ca pantothenate‡	8	”	”
No supplement	10	3	5
0.1 ml corn oil	9	7	2

*National Oil Products Company, Harrison, N. J.
†Equivalent to 100 mg of Eli Lilly liver extract No. 343.
‡Generously supplied by Merck & Co., Rahway, N. J.

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[3] Guerrant, N. B., and Salmon, W. D., *J. Biol. Chem.*, 1930, **89**, 199.

the lumen. A very excellent report of the relation of pantothenic acid to the histopathology of filtrate-factor deficiency has been made by Ashburn.[4]

In conclusion, evidence is presented to confirm the findings of Daft and coworkers that pantothenic acid prevents the adrenal hemorrhage and necrosis frequently observed in filtrate-factor-deficient rats.

11772

Measurement of Diodrast and Inulin Clearances in Man After Subcutaneous Administration.*

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The administration of diodrast and inulin by constant intravenous injection for periods of 2-4 hours has been undoubtedly tedious to many of our patients, has made the use of catheters obligatory, and has required the coöperation of at least 2 operators when blood and urine samples are drawn at frequent intervals. One of us (HLW) found that in dogs subcutaneous injection of diodrast resulted in plasma concentrations sufficiently constant for the purpose of clearance estimation. We have greatly simplified our clinical work by applying this technic to humans.

Technic. Breakfast is withheld and a blood sample drawn for analytical blanks. Under procaine anesthesia a warm, freshly prepared 25% inulin solution (0.2 g inulin/kg) is introduced into the loose subcutaneous tissue of the axilla by means of a 50 cc glass syringe and a long spinal needle. The patient drinks a liter of water. About 45 minutes later diodrast (0.2 cc/kg) is similarly injected through the same wheal; if it is introduced directly into the inulin puddle the diodrast need first be diluted with only 2 or 3 volumes of salt solution in order to avoid irritation. Ten or 15 minutes later the first blood sample is taken, the patient discards his bladder urine, and clearance periods are then obtained in the usual way. Satisfactory plasma concentrations exist for at least 2 hours

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[4] Ashburn, L. L., *Public Health Rep.*, 1940, **55**, 1337.

* Supported by grants from the Smith, Kline and French Laboratories (T.F.) and the Commonwealth Foundation (H.L.W.).

more; we routinely obtain 3 half-hour urine collections. Unless there are special indications for catheterization we rely upon the ability of the average patient to empty his bladder completely when it contains about 200 cc of urine; wide fluctuations in urine volume have no effect on diodrast or inulin clearances.

For an average adult the volume of the combined injection fluids is less than 100 cc. Some patients receive transient discomfort from tissue distention but none, even children, have complained of pain.

Methods. Inulin (Pfanstiehl, C.P.) is washed by boiling in water and reprecipitating with alcohol at least twice; when this was not done, injection caused much residual pain. To the required amount is added enough 0.5% saline to make a 25% solution; this is dissolved by boiling, filtered through paper, and boiled again for 10 minutes just before use. Diodrast (Winthrop Chemical Co.) is the 35% solution and is quite irritating unless diluted at least 3 times. If hematocrit readings are taken in order to calculate whole blood flows all blood samples are heparinized. Iodine determinations are done either by the original[1] or the modified[2] method of White and Rolf. The inulin method is modified from that of Corcoran and Page.[3] In these preliminary experiments we have not employed yeast fermentation but have used the preinjection blood sample as a blank by regarding its colorimeter reading as the zero point for subsequent determinations. Fluctuations in blood glucose are not hereby accounted for but the error is slight; a change of 50 mg % in plasma glucose corresponds to a change of less than 2 mg % in plasma inulin. Urine blanks are prepared from distilled water. Our acid alcohol-diphenylamine solution is made as follows: 7.5 cc 20% diphenylamine in alcohol, 56 cc 95% alcohol and 62.5 cc concentrated HCl. Three cc of this are added to 1 cc of plasma filtrate ($Zn\ SO_4$-NaOH) or diluted urine, and the tubes are boiled for exactly 15 minutes. They are then quickly cooled in ice for 5 minutes and the volume of each tube made up to 7 cc with 95% alcohol. Readings are made in a Klett photoelectric colorimeter with a 620 filter against a curve prepared from known levulose solutions. This modification permits satisfactory analyses on plasma or diluted urine containing 10-30 mg % inulin.

Results. Table I gives the essential data on 3 hypertensive individuals examined in this way. All had urea clearances of at least 50% of normal so that the sustained blood concentrations of diodrast

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[1] White, H. L., and Rolf, D., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 1.

[2] White, H. L., and Rolf, D., *Ibid.*, 1940, **45**, 433.

[3] Corcoran, A. C., and Page, I. H., *J. Biol. Chem.*, 1939, **127**, 601.

TABLE I.

Period	Time in min	Urine flow cc/min	Diod. Iodine mg %		Inulin mg %		Plasma Diodrast Clear	Plasma Inulin Clear
			Plasma	Urine	Plasma	Urine		
Patient RD								
I	31	10.7	3.6	82.8	22	127	258	65
II	19	7.6	3.88	131	28.8	230	268	64
III	30	4.27	3.57	186.4	30	384	234	57
Patient GW								
I	27	5.11	3.28	204	16	227.5	296	68
II	30	7.53	3.6	145.2	18.2	184	283	71
III	31	7.36	3.3	124.2	19.3	202	258	72
Patient MC								
I	30	2.74	2.13	316	14	450	463	100
II	23	3.87	1.94	198	17	397.6	450	103
III	16	6.88	1.82	95	19.9	270	408	106

and inulin are not due to defective renal function; curves on normal subjects are essentially similar. All of the urine specimens were voided and the patients were more or less ambulatory throughout the observation periods. The plasma values are interpolated to the mid-period from actual determinations made before the beginning of Period I, during Period II, and after the end of Period III. Clearances are in terms of cc/min/1.73 sq meters.

Patient RD was also examined by the intravenous technic, the urine being collected by bladder washings through a catheter. Consecutive clearances were as follows: diodrast (274, 264, 270); inulin (68, 66, 74).

Summary. Satisfactory plasma clearances of diodrast and inulin can be obtained in humans following subcutaneous injection of the respective substances.

11773 P

Reproduction in Rats on Synthetic B-Complex Supplement.*

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Several members of the vitamin B complex are now available in synthetic form. In a search for unidentified nutritional factors, the following diet (diet R-1) was fed to black rats: sucrose, 60 g;

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* Aided in part by a grant from the Williams-Waterman Fund of the Research Corporation, New York.

casein, acid-washed, 25 g; hydrogenated cottonseed oil ("Crisco"), 8 g; salt mixture,[1] 5 g; fish oil blend (3000-A, 400-D), 0.3 g; choline chloride, 50 mg; pantothenic acid, 2.8 mg;† nicotinic acid, 1.0 mg; riboflavin, 0.5 mg; thiamin, 0.2 mg; pyridoxine, 0.2 mg.‡ In addition, the rats received a weekly dose of 0.5 cc of corn oil mixed with 0.05 cc of shark liver oil containing 200,000 units of vitamin A per gram. In this diet, the fat-soluble factors are fed in a crude form, but the water-soluble factors are all synthetic and are fed at levels which were thought to be more than sufficient in each case. Nicotinic acid is not known to be needed by rats, but was fed as a precautionary measure. Growth on diet R-1 took place at about 60% of the normal rate for the strain for the first month, but at 80 days the rats had attained about 80% of normal weight.

In the first experiment 4 female rats were placed on diet R-1 at weaning (19 days of age). After 76 days they were mated to males which had been reared on a stock diet. Three litters of 8, 6, and 6 offspring respectively were produced and were continued on diet R-1. Seven rats now survive from these litters and averaged 160 g in weight at 80 days. Six of the rats appeared normal and had black hair. The seventh, a male, was slightly grey. The young in this and the succeeding experiment were about 60% of normal weight at birth.

In the second experiment, 2 groups of female rats were placed on diet R-1 at weaning and at an age of 113 to 153 days were mated with males which had also been on diet R-1 since weaning. One cc of wheat germ oil was fed to each female after mating. The first group consisted of 6 females and was continued on diet R-1 through pregnancy. Five litters, totalling 37 young, were produced. Three of these litters, totalling 22 young, survived and appeared to be developing normally at one week of age. The second group consisted of 9 females and was placed at mating on a diet which contained no pantothenic acid, but otherwise was identical with R-1. The 3 females which had produced litters in the first experiment were included in this group. Three rats in this group resorbed, and 5 of the others produced litters, totalling 28 young. The young were apparently healthy, but suckling was never observed, and they all died within 4 days of birth.

The feces of the rats on diet R-1 were small and black, which did not indicate refection. Greying has not been observed in the female rats, but 5 males, averaging 394 g in weight, showed definite greying at 163 days of age after being kept on the diet since weaning. The high mortality of the young may have been due partly to injuries received from the wire floors during the first few days. Diet R-1 permits some degree of reproductive success, but is obviously not as good as a "normal" diet of natural foods. It is of interest, however, to record the rearing of healthy representatives of a second generation of rats on a diet supplemented with the B-vitamins in a synthetic form.

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[1] Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 180.

† Prepared by Dr. S. H. Babcock.

‡ 2-methyl 1,4-naphthoquinone diacetate, 0.04 mg, has recently been included. This addition was made after an entire litter of "second generation" rats bled to death upon being earpunched at the age of 3 weeks. No tendency to prolonged bleeding has been observed since vitamin K has been added to the diet.

11774

Purification of Follicle-Stimulating Hormone (FSH) of the Anterior Pituitary.*

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Purification of the FSH has been achieved by the following steps: (1) extraction of acetone desiccated sheep pituitaries with 40% alcohol; (2) precipitation from 50% acetone at pH 4.5; (3) ammonium sulphate (A.S.) fractionation in which the hormone is reprecipitated several times between 0.5 and 0.67 SAS; (4) precipitation from 33% acetone at pH 4.8; (5) precipitation from 67% alcohol; (6) removal of less active material by saturation with NaCl at pH 4.1.

Details of this procedure have been published.[1] The product contained 1 unit in 6 γ (for method of assay and definition of unit, see below). It has recently been found that fractionation of very dilute FSH solutions (0.075%) with A.S. generally leads to doubling of hormonal activity in a fraction precipitating between 0.6 and 0.7 saturation with A.S. (the protein concentration at this stage being

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* Aided by grants from the Board of Research of the University of California, the Rockefeller Foundation, New York City, and Parke Davis and Company, Detroit, Michigan. We wish to acknowledge assistance from the Works Progress Administration, Project No. O. P. 65-1-08-62, Unit A-5.

[1] Fraenkel-Conrat, H., Simpson, M. E., and Evans, H. M., *Anales de la facultad de Medicina, Montevideo,* 1940, XXV.

TABLE I.
Standardization in Hypophysectomized Rats of an FSH Preparation Containing 1 Unit in 3 Gamma.

		Ovaries		
Total dose, γ/R	No. of rats	Wt, mg	Follicles (Histology*)	Estrous uterus No. of rats
18	8	39	many medium large	4
15	8	30	medium large	1
12	3	33	small, medium, few large	0
9	3	25	small, medium	1
6	3	22	” ”	0
5	4	22	” ”	0
4	6	15	few small medium	0
3	9	14	many small, few small medium	0
Controls	100	11	small only	0

*Interstitial tissue is deficient in all cases.

about 0.02%). Fractions precipitating below 0.6 and above 0.7 saturation show potencies similar to or lower than those of the material before fractionation. By this method preparations can be obtained which contain 1 unit in 2.5-3 γ (or 300-400 units per mg).

When assayed in a 3-day test in immature female rats, such FSH fractions:

1. Injected subcutaneously into hypophysectomized animals cause follicular development associated with increase in ovarian weights. The definition of the FSH unit is based on this effect; 1 unit represents the minimal total dose which will cause the resumption of follicular growth with antrum formation (Table I). There is no alteration of the interstitial tissue which consists of so-called "deficiency cells" as in uninjected hypophysectomized controls of the same postoperative period.

2. When the injections are made intraperitoneally into hypophysectomized rats, 15 FSH units will bring about beginning luteinization of follicles whereas repair of the interstitial tissue begins only on injection of 40 units and is not complete until 200 units are injected. Subcutaneous injections produce these secondary effects only at somewhat higher dose levels.

3. On subcutaneous injection into normal immature rats, 3-4 units are required to cause a detectable ovarian or uterine stimulation; on intraperitoneal injection about 6 units show an effect.

4. In both normal and hypophysectomized rats about 4-6 times the minimal dose causes the appearance of estrous uteri. In both types of rats, ovarian weights rarely exceed 60 mg.

5. On subcutaneous injection of FSH simultaneously with chorionic gonadotropin, "augmentation" is observed, *i. e.,* ovarian weights are increased considerably beyond what would be expected

as an additive effect of the two hormones. This effect is used as an alternate assay method. One hypophysectomized rat unit was found to cause 100% increase in the ovarian weights of normal rats over those produced by a standard amount of chorionic gonadotropin (8 R.U.); this was defined as 1 Augmentation Unit (Table II). Some degree of augmentation can be obtained regardless of whether the two hormones are injected mixed or at different sites of the body and the phenomenon is therefore not due to delay of absorption or similar nonspecific means of augmenting gonadotropic reactions.

6. On intraperitoneal injection in normal rats low levels of FSH (1-6 units) decrease the effect of other gonadotropins, *e. g.*, that of pregnant mare serum.[2] This antagonistic effect is also given by 1 unit of the other pituitary gonadotropin, ICSH. It appears, however, that it represents an independent property of each pituitary gonadotropin, since antagonism can be elicited with amounts of FSH which are very much smaller than those necessary to cause interstitial cell stimulating effects.

The physico-chemical properties of the most active FSH preparations correspond well to those ascribed by others to less active

TABLE II.
Standardization of Purified FSH Preparation in Normal Immature Female Rats. By "augmentation" of effect of a standard amount of chorionic gonadotropin (C. G.).

	Fraction					
	IV F102A I		IV F102B II		IV F102E II	
Total dose, γ/rat	No. of rats	Wt of ovaries mg	No. of rats	Wt of ovaries mg	No. of rats	Wt of ovaries mg
12					6	126
10	6	100				
9					9	107
8	3	113	3	149	9	93
6	3	118	6	114	18	99
5	9	112	6	118	9	87
4	9	85	6	98	15	79
3	6	70	6	88	18	73
2.5	6	66	6	60		
2.3					6	57
2.0			6	42	3	60
Control (C.G. alone)	12	32	18	34	36	36
1 unit (= 100% augmentation of the effect of C.G. alone)	2.5 γ		2.7 γ		3 γ	

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[2] Fraenkel-Conrat, H., Simpson, M. E., Li, C. H., and Evans, H. M., *Anales de la facultad de Medicina, Montevideo,* 1940, XXV.

preparations.[3, 4, 5] A preliminary electrophoresis experiment has shown FSH to consist mainly of one component.

Summary. The method of preparation of FSH in highly potent form is outlined. Some of the biological properties of this fraction are described.

11775

Eating Habit and Fasting Metabolism of Rats.

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The rate of fasting metabolism depends on the level of previous food intake. In order to measure the influence on the metabolic rate of any condition which lowers the appetite one must therefore reduce the food intake of controls to that of the experimental animals in order to eliminate the influence of food level. The rats whose food intake is thus reduced but whose appetite is not affected may eat their daily ration at once while their pair mates with their decreased appetite on unlimited food supply extend their food intake over the whole day.

Table I shows the feeding habit of 18 rats during 12 days. The amounts eaten during the various daily periods are expressed in percent of the daily intake. The behavior of 9 rats that had pre-

TABLE I.
Eating Habit of Rats.
Means of 9 rats.

Nutritive condition of rats	Food eaten per day per rat g	Food eaten in % of daily intake in following periods				
		4-8 p.m. %	8-12 p.m. %	0-8 a.m. %	8-12 a.m. %	0-4 p.m. %
A. Mean of 12 days:						
1. Previously on unlimited food	14.9	16	23	47	10	4
2. ’’ ’’ restricted ’’	15.2	26	25	30	15	4
B. 1st day after on day’s fast:						
1. Previously on unlimited food	15	60	7	20	0	13
2. ’’ ’’ restricted ’’	24	71	8	4	4	13

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[3] Greep, R. O., van Dyke, H. B., and Chow, B. F., *J. Biol. Chem.*, 1940, **133**, 289.

[4] Fevold, H. L., Lee, M., Hisaw, F. L., and Cohn, E. J., *Endocrinology*, 1940, **26**, 999.

[5] Jensen, H., Tolksdorf, S., and Bamman, F., *J. Biol. Chem.*, 1940, **135**, 791.

viously been on a restricted ration was essentially the same as that of their controls whose food intake had never been restricted. The previously restricted rats ate, however, during the first 4 hours of unlimited food supply 17 g of food which is more than their later average food intake during an entire day and is 71% of the intake during the first day of unrestricted intake. Does such a difference in the distribution of a given daily ration during the day affect the subsequent fasting metabolism? If such an effect would be found then it would not be sufficient to equalize the daily ration between experimental animals and controls but it would be necessary also to parallel with the controls the feeding habit of the experimental animals.

Method. Six female rats (Long-Evans strain) were given 8 g per day per rat of a food mixture which is considered qualitatively complete. Three of these rats received this ration at one time, at 4 p. m. The other 3 were given their food in 5 portions of 1.6 g at 4 p. m., 8 p. m., 12 p. m., 8 a.m., and 12 noon. Four days after the start of this regime, at the age of 88 days, the fasting metabolism of the 6 rats was measured by a respiration trial. After this trial the type of feeding was reversed. Those rats that had been fed once a day were now given their food in 5 portions and the others were now fed once per day, the total food intake remaining 8 g per day. The fasting metabolism was measured again at the age of 94 days.

The details of our respiration apparatus and the standard procedure of these trials are described elsewhere.[1] At 5 p. m. on the day prior to the respiration trial the rats were brought with water but without food to an air conditioned room kept at 30°C. The next day at 8 a. m. the rats were transferred to the multiple respiratory cabinet which was also kept at 30°C Oxygen consumption was read at half-hour intervals and CO_2 production determined at the end of the 4-hour respiration trial.

Results. Table II shows that the metabolic rate of the 6 rats is practically the same whether they have been fed their daily ration of 8 g food once per day or in 5 portions of 1.6 g each. The difference between the 2 mean results for the daily heat production per unit of the 3/4 power of body weight in kilograms, namely 78.3 ± 2.3 and 78.2 ± 2.3 kcal/kg$^{3/4}$ has a standard deviation of ± 3.2 kcal/kg$^{3/4}$. The difference is thus only 1/30 of its standard deviation, it would not be regarded as significant even if it were 60 times as great as it is One reason for this small difference is

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[1] Kleiber, M., *Univ. Calif. Publ. Physiol.*, in press.

TABLE II.
Effect of Once- vs. 5-Times Feeding per Day on Fasting Katabolism of Rats.

	Mean age days	Mean body wt g	Mean heat production per day per kg¾ kcal
6 rats fed 8* g per day once	91	161	78.3 ± 2.3
Same rats fed 1.6 g per day 5 times	91	162	78.2 ± 2.3

*N.B. These rats fed *ad lib.* ate 24, 14, 15, and 14 g of the same food on the first to the 4th day respectively after the respiration trial.

probably our method of using each rat for each of the two conditions, thus eliminating the influence of individual variation between rats on the difference between the means. Our trial shows that this individual variation in the metabolic rate between different rats is much greater than a difference resulting from a change in the number of daily feedings. For the measurement of the fasting metabolism it is thus sufficient to equalize the previous daily rations between experimental and control rats without regard to their feeding habit.

11776

Studies of Phenothiazine. X. Further Observations on Oxidation of Phenothiazine.*

FLOYD DEEDS.

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Several years ago an interesting observation was made on the oxidation of phenothiazine in the dry state. At that time no practical importance was attached to the observation, and it was not reported. As will be shown later, recent developments on the use of phenothiazine as a fungicide have pointed to the importance of this observation, which has now been repeatedly confirmed, and deemed worthy of a report.

A porcelain mortar and pestle had been used for intimately mixing a small quantity of phenothiazine with the basic rat diet routinely used in this laboratory. Subsequently the same mortar was wiped out, but not washed, and used for mixing bentonite with rat diet. It was observed that after a few minutes of mixing the whole con-

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* Food Research Unit Contribution No. 507.

tents of the mortar had assumed a pink tint. Realizing that traces of phenothiazine might have been left in the mortar, and believing that an oxidation to thionol could account for the color developed, the following experiments were performed.

The mortar and pestle were thoroughly washed, and a sample of bentonite alone was thoroughly ground. No coloration developed. After washing the mortar and pestle, phenothiazine alone was ground and again no color developed. The experiment was then repeated with a mixture of phenothiazine and bentonite. After a few moments of grinding the mixture showed a definite pink color which rapidly developed into a deep brick-red as the grinding proceeded. Repetition of the experiment by other members of the staff yielded the same result. Substitution of talc for bentonite gave the same results, but the color development was somewhat slower, indicating that talc was less effective than bentonite in facilitating the oxidation of phenothiazine.

To determine whether or not the grinding was an essential part of the oxidation mechanism, a sample of bentonite and phenothiazine was placed in a test tube and the contents were mixed by repeated but gentle inversions of the tube. In the course of several hours a definite degree of color was observed. The tube was stoppered and set aside. The color continued to deepen and after several weeks was grey-black. Apparently grinding is desirable only insofar as it facilitates the process.

Twenty-five grams of phenothiazine and 100 g of bentonite were placed in a ball bill and ground for 48 hours. Within a few minutes after starting the ball mill, a pronounced brick-red color developed. The mill was stopped and a sample of the mixture removed. Attempts to remove a colored product from the mixture by extraction with water, or by extraction with ether and chloroform, gave negative results. After 48 hours of grinding the mixture was nearly black. From this mixture a red dye could easily be extracted with alcohol, ether or chloroform. When a chloroform extract was filtered to remove bentonite, and evaporated to dryness, the residue consisted of a mixture of a reddish-black powder and a light-colored powder which was probably unchanged phenothiazine. This residue was completely soluble in alcohol, but upon the addition of water the solution yielded a fine silky precipitate characteristic of phenothiazine. Removal of this precipitate left a clear, light-red aqueous solution. Chloroform extraction of this solution permitted recovery of a phenothiazine-free dye. An aqueous solution of this dye exhibited reactions characteristic of thionol. It was easily reduced by stannous chloride and sodium hydrosulfite and reoxidized by hydrogen peroxide, or simply by shaking during exposure to air.

Further identification of the dye as thionol has not been attempted, nor have tests been made for the presence of the intermediate oxidation product, namely, phenothiazone.

The results obtained suggest that the oxidation of phenothiazine which normally takes place slowly in the presence of moisture, light, and air is greatly facilitated by the distribution of the phenothiazine over the extensive surface present in finely divided bentonite. That this is a fairly general phenomenon is shown by preliminary experiments in which dimethylacridan and diphenylamine have been oxidized to colored compounds by grinding with bentonite.

If compounds of low-water solubility are of insecticidal and fungicidal value by virtue of slow oxidation to a more potent compound, it may well be that a mixture of these compounds with bentonite will improve their efficiency.

Recently, M. C. Goldsworthy and E. L. Green of the Bureau of Plant Industry, U. S. Department of Agriculture, claimed to have discovered that the fungicidal action of phenothiazine is due to its partial oxidation to phenothiazone, according to the following statement quoted from U. S. Department of Agriculture press release dated February 11, 1940:

"An attempt has been made to find a way to spray the chemical so that some of it would change to phenothiazone and kill fungi and the remainder keep its original form as an insect killer. Some promising results were obtained by mixing phenothiazine with bentonite—a fine clay—and hydrated lime. This caused the spray to stick to the fruit and build up a residue. The under part of the spray film apparently retains its insecticidal value while that exposed to light on the surface of the film changed to a fungicide."

The experiments reported here suggest that the distribution of phenothiazine over the extensive surface presented by bentonite permits intimate contact with atmospheric oxygen, thereby facilitating oxidation, an action which may be further aided by the alkalinity of the hydrated lime.

11777 P

Electrophoretic Analysis of Digested Antitoxic Sera.

J. VAN DER SCHEER AND RALPH W. G. WYCKOFF.
From the Lederle Laboratories, Inc., Pearl River, N. Y.

Recent work has shown that certain antitoxins can be partially digested[1] with pepsin without losing their abilities to neutralize toxin. The chemical changes[2] that accompany this alteration are apparently small; the immunological modification[3] is, however, clearly set forth in the seriously impaired antigenicity of digested antitoxins. We have made electrophoretic studies of a number of such digested antitoxic sera. In this way it has been possible to follow the course of the enzymatic digestion and to obtain an objective record of the changes that occur when sera are modified.

In these experiments diphtheria and tetanus antitoxic horse sera have been digested with pepsin at 37°C and pH 4. Electrophoretic analyses have been made of the original sera and of samples of the digest mixture taken at various intervals during the digestion process.

Previous work has shown that powerfully antitoxic horse sera contain large new T-components[4] which develop during the course of hyperimmunization. Such a T-peak appears in the electrophoretic pattern of the diphtheria and tetanus antitoxic sera shown

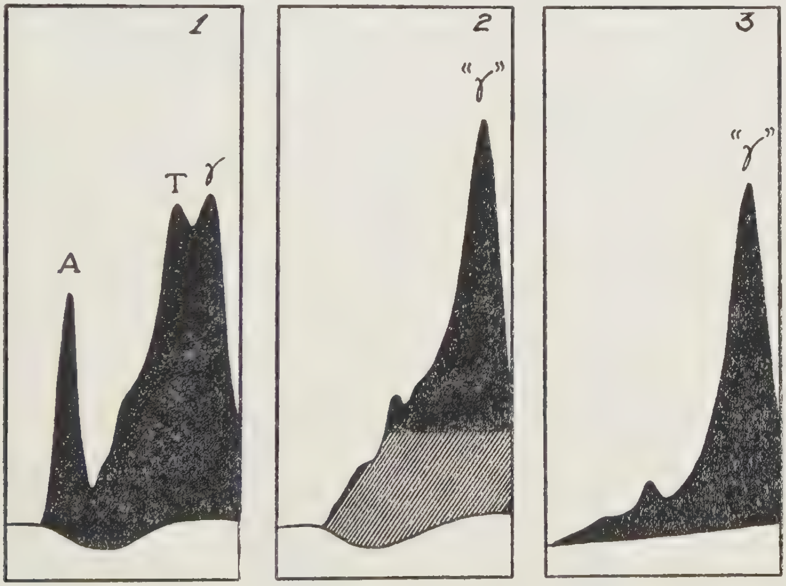

FIG. 1. Electrophoretic diagram of a defibrinated diphtheria antitoxic horse plasma (No. 4635) diluted 1:4 and dialyzed against pH 7.6 buffer-saline.

FIG. 2. Electrophoretic diagram of plasma No. 4635 after one-half hour's digestion.

FIG. 3. Diagram of plasma No. 4635 after digestion for 100 hours.

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[1] Parfentjev, I. A., U. S. Patents No. 2065196 and No. 2123198.

[2] Pope, C. G., *Brit. J. Exp. Path.*, 1938, **19**, 245; 1939, **20**, 132, 201.

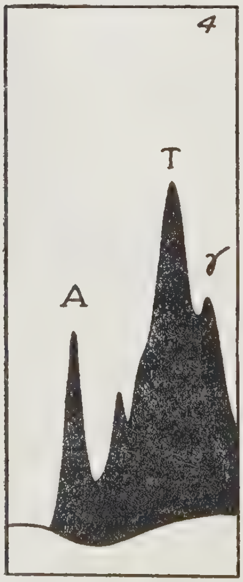
[3] Weil, A. J., Parfentjev, I. A., and Bowman, K. L., *J. Immunology*, 1938, **35**, 399.

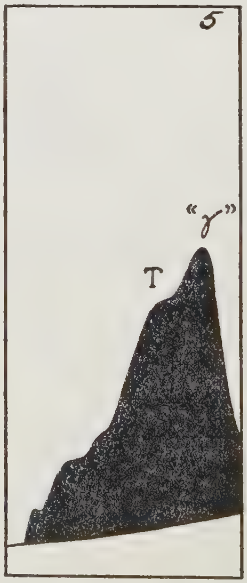
[4] van der Scheer, J., and Wyckoff, R. W. G., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 427; van der Scheer, J., Wyckoff, R. W. G., and Clarke, F. H., *J. Immunol.*, 1940, **39**, 65.

in Figs. 1 and 4. A study of areas and mobilities in the electrophoretic diagrams demonstrates that during digestion this T-component is more or less rapidly converted into a slower component that moves at substantially the same rate as the normal γ-globulin. As can be seen from Fig. 2 no T is left in a diphtheria antitoxin after one half hour's digestion. A great advantage of digested antitoxin lies in the comparatively slight reactions that follow its injection into man; in order to modify it to this extent, however, a serum must be digested for many hours. Loss of reactivity is not therefore the immediate consequence of the transformation of the T-globulin. A comparison of Fig. 2 and 3 shows that prolonged digestion does not further alter the mobility of the transformed T component but it does eliminate electrophoretically-heterogeneous material such as that which has been shaded in Fig. 2. It is not improbable that reactivity on injection is associated with this heterogeneous protein.

Steps in the conversion of the T-globulin can be seen in Figs. 4-6. After only 10 minutes' digestion (Fig. 5) some, but not all, the T has disappeared; at the end of 30 minutes it has been completely transformed (Fig. 6).

In a later publication we shall discuss in greater detail the electrophoretic evidence concerning the action of pepsin on immune sera and shall compare this evidence with measurements of the loss of coagulable protein and of antibody activity during digestion.





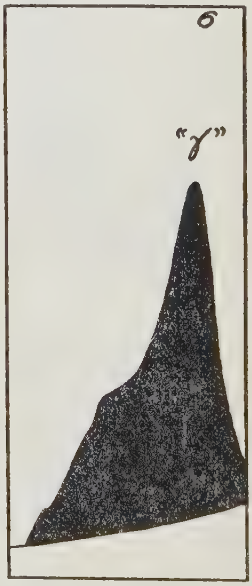


FIG. 4. Diagram of a tetanus antitoxic horse serum No. A787.
FIG. 5. Diagram of serum No. A787 after 10 minutes' digestion.
FIG. 6. Diagram of serum No. A787 after one-half hour's digestion.

11778

Studies With Antigens, VIII. Preparation of Purified Extracts of Ragweed Pollen.

C. H. Boatner, B. G. Efron and M. R. Pabst.
(Introduced by Isidore Cohn)

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We have described[1, 2, 3] the preparation of purified house-dust and grass-pollen extracts of higher purity, higher concentration, and greater allergenic activity than extracts prepared by previous methods.

The purpose of the present investigation is to apply the technic used in the preparation of purified house-dust and of purified grass-pollen extracts to the preparation of highly purified extracts of ragweed pollens. The pollens of *Ambrosia artemisiaefolia* and *Ambrosia trifida* were studied.

Aqueous extracts of defatted pollens were subjected to fractional precipitation by the addition of water-miscible organic liquids such as dioxane, acetone and isopropyl alcohol. The fractionation technic is briefly described as follows: The precipitate which formed when a small amount of the organic liquid was added to the aqueous extract was removed, then an additional amount of organic liquid was added to the filtrate; this procedure was repeated to give progressively larger proportions of organic liquid with successive separations of insoluble fractions. The fractionations with organic liquids were carried out at low temperatures. Fractionations of aqueous ragweed pollen extracts with dioxane and other organic liquids resulted in the separation of the solutes into 3 fractions, one only slightly and the other 2 completely soluble in water.

For purposes of clarity, our description will be limited to the results obtained by the use of dioxane.

Dialysis experiments showed that No. 1200 cellophane and Visking Standard Stretch Membrane were semi-permeable and permitted the passage of dialyzable material possessing very little skin reacting potency per unit weight of dissolved material in the dialysates. Dialysis was carried out at low temperatures.

Original aqueous extracts and aqueous solutions of extracts frac-

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[1] Boatner, C. H., Efron, B. G., and Dorfman, R. I., *Science*, 1940, **91**, 389.

[2] Boatner, C. H., Efron, B. G., and Dorfman, R. I., *Allergy*, in press.

[3] Boatner, C. H., and Efron, B. G., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 460.

tionated with dioxane were treated with high concentrations of sulphate salts: namely, ammonium, sodium, and zinc sulphate. In the use of ammonium sulphate, 60 g of salt were added to each 100 ml of the extract. The precipitate which formed was removed and treated with water. The resulting solution was cleared and was again treated with ammonium sulphate. The precipitate was removed, was treated with a small volume of water, and the resultant solution was dialyzed against running water until it was freed of sulphate.

Typical results are shown in Tables I and II. The comparative scratch tests[4, 5] were made with equal concentrations of extract on clinically sensitive patients.

The skin reacting potency of original ragweed extracts was found to be significantly greater per unit weight of material than that of the fractions precipitated by low concentrations of dioxane (Table I, 1). The skin reacting potency of the fraction precipitated by the intermediate concentrations of dioxane was significantly greater per unit weight of dissolved material than either that of the original extract (Table I, 2) or that of the fraction precipitated by high concentrations of dioxane (Table I, 3).

The skin reacting potency of the fraction which was precipitated by the intermediate concentrations of dioxane and which was then

TABLE I.
Fractionation with Dioxane.

Extracts	No. equal size reactions	No. smaller reactions	No. larger reactions	Total	P*
1. Original	2	0	18	20	<.01
From low conc. dioxane	2	18	0	20	
2. Original	17	52	7	76	<.01
From intermediate conc. dioxane	17	7	52	76	
3. From high conc. dioxane	16	26	5	47	<.01
From intermediate conc. dioxane	16	5	26	47	
4. Original	32	68	22	122	<.01
Fractionated with dioxane, dialyzed	32	22	68	122	
5. Original, dialyzed	26	30	8	64	<.01
Fractionated with dioxane, dialyzed	26	8	30	64	

*The probability is approximated by the Chi-Square Test.[6]

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[4] Efron, B. G., Boatner, C. H., Pabst, M. R., *Int. Corresp. Club of Allergy*, 1940, **3**, 59.

[5] Pabst, M. R., Boatner, C. H., Efron, B. G., *New Orleans Med. and Surg. J.*, 1940, **93**, 142.

[6] Fisher, R. A., *Statistical Methods for Research Workers*, 7th Ed., Oliver and Boyd, London, 1938.

TABLE II.
Fractionation with Ammonium Sulfate.

Extracts	No. equal size reactions	No. smaller reactions	No. larger reactions	Total	P*
1. Fractionated with dioxane, dialyzed	8	21	3	32	<.01
Fractionated with dioxane, $(NH_4)_2SO_4$, dialyzed	8	3	21	32	
2. Original	19	93	12	124	<.01
Fractionated with dioxane, $(NH_4)_2SO_4$, dialyzed	19	12	93	124	
3. Original, dialyzed	11	27	8	46	<.01
Fractionated with dioxane, $(NH_4)_2SO_4$, dialyzed	11	8	27	46	
4. Original, fractionated with $(NH_4)_2SO_4$, dialyzed	18	26	11	55	.02-.01
Fractionated with dioxane, $(NH_4)_2SO_4$, dialyzed	18	11	26	55	

*Footnote, Table I.

dialyzed was significantly greater per unit weight of dissolved material than that of the original extract from which it was processed (Table I, 4). It was also significantly greater than that of the original extract which had been dialyzed (Table I, 5). The dialyzed extracts compared had been subjected to dialysis against running water through similar membranes over the same period of time.

The skin reacting potency of the extract treated by fractionation with dioxane and with ammonium sulphate, and then by dialysis was significantly greater per unit weight of dissolved material than that of the extract purified by fractionation with dioxane and by dialysis (Table II, 1). The skin reacting potency of the former fraction was also significantly greater per unit weight of dissolved material than that of the original extract from which it was processed, than that of the original extract after it had been dialyzed, and than that of the original extract treated with ammonium sulphate and dialyzed (Table II, 2, 3, 4). In each instance in which dialyzed extracts were compared, they were subjected to dialysis against running water through similar membranes over the same period of time.

The increased skin reacting potency of the processed ragweed pollen extracts is due to the purification of the allergens resulting in their increased concentration per unit weight of dissolved material in the extracts. This conclusion is warranted by the following observations: Scratch tests performed on individuals who had no allergic disease failed to show any evidence of irritation when tests were made with the various fractions of the ragweed extracts. Patients who suffered from allergic disease but who were not clinically sensitive to ragweed pollens failed to show positive reactions to scratch tests with the various fractions. Patients who were clinically sensitive to ragweed pollen showed significantly larger skin reactions with the more highly purified extracts than with the less highly purified extracts or with the untreated extracts.

Summary. 1. Fractionation of aqueous ragweed pollen extracts with water-miscible organic liquids produces purification and concentration of the allergens of these extracts. 2. Fractionation of extracts, purified by the above treatment, with high concentrations of sulphate salts produces further purification and concentration of the allergenic factors.

11779

Changes in Blood Ketone Acids During Artificial Fever.

MICHAEL SOMOGYI AND MELVIN B. KIRSTEIN.
From the Laboratory of the Jewish Hospital of St. Louis, Mo.

Fever, whether infectious or artificial in origin, increases the metabolic rate and consequently influences carbohydrate metabolism. The initial effect is an increased utilization of carbohydrate in the tissues, resulting in an accelerated withdrawal of glucose from the blood. As a means of compensating for the drain on blood sugar, a rise in the rate of hepatic glycogenolysis takes place, and this in turn leads in the postabsorptive state (or when glucose is absorbed at a lower rate than it is removed from the blood) to a depletion of the glycogen stores of the liver.

Observations on patients and on laboratory animals, undergoing treatment with hyperthermia, clearly show the process in its several phases. In a previous communication[1] we pointed out the fact that the hyperglycemia that occurs during fever is due to excessive hepatic glycogenolysis, and not to blood concentration, as several workers believed. In accord with this view, von Haam[2] and Gunderson and

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[1] Kirstein, M. B., and Bromberg, L., *J. Lab. and Clin. Med.*, 1939, **25**, 7.

[2] Von Haam, E., Changes in the parenchymatous organs and blood vessels produced by artificially induced fever. Presented at conference on fever therapy, under auspices of the Kettering Foundation, St. Louis, Mo., Nov. 11, 1938.

Loseke[3] found a marked depletion of the glycogen stores in the livers of laboratory animals that were subjected to prolonged and severe hyperthermia.

Since the production of ketone bodies is intimately connected with carbohydrate metabolism, it seemed of interest to investigate possible changes in ketonemia during artificial fever. We studied 6 patients, 3 males and 3 females; 5 of these were afflicted with syphilis of the central nervous system, the sixth, an 11-year-old girl, had Sydenham's chorea. By performing glucose tolerance tests on the patients, we ascertained that their carbohydrate metabolism was normal.

In every treatment fever was maintained for 4 hours at 40 to 41° (rectally measured). Prior to and between treatments the patients received unrestricted diets; on the morning of treatment they were given 200 cc of orange juice, one cup of coffee with sugar and one slice of toast (about 45 g of carbohydrates). During hyperthermia they drank saline in quantities sufficient to forestall dehydration.

The total ketone bodies in the blood were determined at the onset and at the termination of fever. The analytical method employed was Hubbard's micromethod[4] with some unessential modifications. The maximum error in recovering known quantities of ketone bodies, and in duplication of results by this technic, is less than 10%. The figures, recorded in Table I, show that 4 hours of fever caused a significant rise in ketonemia in all of our subjects; the increase varied in 5 cases from 80 to 140%, and in the sixth case it rose to 14-fold of the initial level.

This phenomenon can be readily understood, and even anticipated, if one postulates as satisfactorily proved 2 observations of Embden, *et al.*, first, that the liver is the sole source of the ketone bodies

TABLE I.
Effect of Artificial Fever upon Ketonemia.

			Ketone bodies in blood, expressed as β-hydroxybutyric acid		
Patient	Sex	Age	Before treatment mg %	At end of treatment mg %	Increase %
P.L.	F	28	1.08	2.34	116
N.W.	F	46	0.96	1.73	80
R.C.	M	32	1.10	2.70	145
F.B.	M	26	1.04	1.87	80
M.B.	F	11	0.95	1.93	103
E.E.	M	33	0.47	6.60	1300

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[3] Gunderson, M. F., and Loseke, L. L., Depletion of liver glycogen under experimental fever induction in rabbits. Presented at conference on fever therapy, under auspices of the Kettering Foundation, St. Louis, Mo., Nov. 11, 1938.

[4] Hubbard, R. S., *J. Biol. Chem.*, 1921, **49**, 375.

that occur in blood and urine,[5] and second, that an inverse relationship obtains between the glycogen content of the liver and the rate of formation of ketone bodies.[6]

The discovery of these facts grew out of perfusion studies of liver and other organs by Embden and his collaborators. Subsequently, numerous other workers, employing improved analytical technic and a wide variety of experimental approaches, concurred without a single exception in Embden's conclusions.[7–13]

Pursuing this concept, it should be possible to prevent, or at least to mitigate, the ketogenic effect of fever by stocking the liver with substantial amounts of glycogen directly before or during fever. Subject E. E. was selected for a series of experiments to probe the validity of the theory; 3 observations were made on him at weekly intervals. First he was treated in the postabsorptive state, 14 hours after his last meal. As may be seen in Table II, the ketone content of his blood rose to nearly 14-fold of the initial level. One week later the treatment was repeated; this time the patient was given breakfast and, in addition, 100 g of glucose intravenously. The injection was started 5 minutes after fever had begun, and was continued for 1 hour and 40 minutes. Hyperthermia was terminated, and a blood sample was withdrawn for analysis, 2 hours and 15 minutes after completion of the glucose infusion.

In this experiment the patient received a total of 145 g of glucose, a substantial portion of which undoubtedly was added to the glycogen stores of the liver. The fever increased the metabolism by about 30% above the normal rate,[14] equivalent to approximately 100 calories for the entire 4-hour period. Even if it is assumed that all of the 100 calories was derived exclusively from glucose, and that the corresponding 25 g of glucose required were derived altogether from hepatic glycogen, the glycogen content of the liver still could scarcely have been lower after than before the fever period. In other words, there is no justification for the assumption that the glycogen stores of the liver were depleted during the treatment. Yet, as may be

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[5] Embden, G., and Kalberlah, F., *Beitr. z. chem. Physiol. u. Path.*, 1906, **8**, 121.

[6] Embden, G., and Wirth, J., *Biochem. Z.*, 1910, **27**, 1.

[7] Burn, J. H., and Marks, H. P., *J. Physiol.*, 1926, **61**, 497.

[8] Raper, H. S., and Smith, E. C., *J. Physiol.*, 1926, **62**, 17.

[9] Chaikoff, I. L., *J. Biol. Chem.*, 1927, **74**, 203.

[10] Edson, N. L., *Biochem. J.*, 1936, **30**, 1862.

[11] Mirsky, I. A., Heiman, J. D., and Broh-Kahn, R. H., *Am. J. Physiol.*, 1937, **118**, 290.

[12] Blixenkrone-Moeller, N., *Z. f. physiol. Chem.*, 1938, **252**, 117.

[13] Harrison, H. C., and Long, C. N. H., *J. Biol. Chem.*, 1940, **133**, 209.

[14] Simpson, W. M., *J. A. M. A.*, 1936, **106**, 246.

TABLE II.
Effect upon Ketonemia of Glucose Injection During Fever Treatment.
These observations were made on patient E.E.

Ketone bodies in blood		Blood sugar		
Before treatment mg %	At end of treatment mg %	Before treatment mg %	At end of treatment mg %	Food intake
				Experiment I
0.53	7.30	69	79	No breakfast, no glucose.
				Experiment II
0.60	3.06	90	66	Breakfast, plus 100 g glucose injected over period of 100 min.
				Experiment III
0.62	0.47	113	162	Breakfast, plus 100 g glucose injected over period of 205 min.

seen in Table II, ketonemia under these conditions has risen to about 5-fold of the initial level. This increase is considerable, even though not quite half as great as it was when the patient received no carbohydrates.

This rise in ketonemia despite the administration of carbohydrate can be explained as follows: During the injection of glucose the blood sugar level was undoubtedly above the postabsorptive level, a condition conducive to glycogen deposition in the liver. Data in hand from other experiments in this laboratory show that ketonemia during such periods declines below the postabsorptive level. Soon after completion of the injection, however, the glycemic level must have rapidly declined in this experiment, dropping below the postabsorptive level and, as may be seen in Table II, remaining low to the end of the treatment. This hypoglycemic interval induced an increase in the rate of hepatic glycogenolysis, and concurrently ketone formation was substantially accelerated. Thus it may be inferred from this experiment that an abnormal increase in the rate of hepatic glycogenolysis *per se* may increase the rate of ketone formation, without the depletion of the glycogen stores of the liver.

The validity of this assumption was tested in a third experiment on the same patient. This differed from the preceding experiment only in one respect, namely, that the injection of the glucose was prolonged, extending over a period of 3 hours and 15 minutes; it was begun 20 minutes after the onset of the fever, and completed 15 minutes before the termination of the treatment. The purpose of this procedure was the production and maintenance of hyperglycemia throughout the fever, designed to compensate for the augmented glucose requirement of the tissues, without a call at any time for an increase in the rate of hepatic glycogenolysis. The data in Table II show that we succeeded in maintaining hyperglycemia to the end of the experiment; concurrently, it may be noted, no rise in the rate of ketone formation has taken place. On the contrary, there was a measurable drop in the ketonemic level.

May it be reiterated that the amounts of intravenously supplied glucose were identical in Experiments II and III (Table II); only the duration of the injection differed. In Experiment II, because of a shorter duration, hypoglycemia was allowed to develop during the latter part of the fever, a factor that invariably increases the rate of hepatic glycogenolysis,[15] while in Experiment III hypoglycemia was avoided by a safe margin throughout the entire experiment, so that the liver had not to be pressed into service to provide for the augmented carbohydrate need of the extrahepatic tissues.

Summary. Artificial hyperthermia in man increases ketosis. This, according to available evidence, is due to an increased rate of ketogenesis in the liver. Our observations on human subjects are in accord with the findings of previous investigators, who demonstrated on perfused liver, on liver slices and on living animals, that an inverse relationship exists between the glycogen content of the liver and the rate of ketone formation. The data presented indicate, moreover, that an increased rate of hepatic glycogenolysis *per se* suffices to increase ketosis, without any appreciable diminution of the glycogen reserves of the liver. An increased ketonemia in artificial fever can be entirely forestalled by the continuous injection of glucose so as to ensure the maintenance of blood sugar at hyperglycemic levels, a condition which enhances glycogen deposition in the liver and at the same time prevents an increase in the rate of glycogenolysis.

11780

Changes in Ketonemia and Ketenuria During Hypoglycemia.

MICHAEL SOMOGYI.

From the Laboratory of the Jewish Hospital of St. Louis, Mo.

Artificial fever, as we reported, causes a substantial rise in ketonemia. Our experimental data indicated that an increase in the rate of hepatic glycogenolysis in itself suffices to accelerate the formation of ketone bodies, irrespective of the glycogen content of the liver.[1] If this concept is correct, then hypoglycemia, which in general entails an increased rate of hepatic glycogenolysis, should cause a rise also in ketonemia.

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[15] Somogyi, M., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 51.

In an inquiry whether or not this is the case, we studied subjects known to develop spontaneously moderate degrees of hypoglycemia during post-absorptive periods. In 3 cases in which hypoglycemia was frequent in the morning hours, we actually found elevated ketone contents in blood samples taken before breakfast. But in these instances one cannot preclude the possibility that the glycogen stores of the liver may have been greatly depleted during the night, due to the possible persistence of the hypoglycemic state over periods of several hours.

This uncertainty was eliminated in experiments in which the subjects were fed 100 g of glucose in the morning, thus providing fresh glycogen reserves for the liver. Individuals with inclination to spontaneous hypoglycemias as a rule develop such states several hours after glucose feedings, and thus one can observe the concurrent changes in blood ketones. Two examples of such experiments are presented in Table I. The subject in the first of the 2 (Case A) was a student, in the second (Case B) a middle-aged woman. In each case 100 g of glucose were given in the morning, and the subjects were at complete rest throughout the experiments. As may be noted in both cases, the blood ketones showed a transitory decrease during the first 3 hours after the ingestion of glucose, but began to rise in the fourth hour, at the time when hypoglycemia had developed. At the end of the fifth hour the ketone body content of the blood had risen to 3.0 and 1.2 mg %, respectively, a level that is well above the normal (0.3 to 1.0 mg %).

TABLE I.
Increase in Ketonemia After Hypoglycemia During Glucose Tolerance Tests.

Hr after ingestion of 100 g of glucose	Subject A Mg in 100 cc of blood Glucose	Subject A Mg in 100 cc of blood Ketones*	Subject B Mg in 100 cc of blood Glucose	Subject B Mg in 100 cc of blood Ketones*
0	66	—	75	0.40
1	131	0.81	—	—
2	81	0.57	80	0.28
3	68	0.44	62	0.31
4	53	0.48	49	0.16
5	66	3.00	78	1.22

*Expressed in terms of β-hydroxybutyric acid.

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[1] Somogyi, M., and Kirstein, M. B., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 640.

TABLE II.
Ketonuria Due to Spontaneous Hypoglycemia (''Hyperinsulinism''?).

Day and hour of observation 1938	Excreted in urine				Remarks
	Glucose g	Ketone bodies		Nitroprusside test	
		mg %	mg/hr		
Feb. 3 and 4					At 9:30 p.m. hypoglycemic symptoms, 40 g glucose given. Symptoms persisted, blood sugar at 10:30 p.m. only 65 mg%. At 11:00 p.m. 50 g glucose given, at 11:30 hypoglycemic reaction marked, 40 g glucose given, followed by 180 g more (part intravenously) to 3:30 a.m., when relief from hypoglycemic symptoms was evident.
5:00 p.m.- 9:30 p.m.	0	....	....	—	
9:30 '' -11:30 ''	0	10	2.5	+	
11:30 '' -12:30 a.m.	0	37	28	+++	
12:30 a.m.- 3:30 ''	0	....	....	+	
3:30 '' - 6:30 ''	0.4	....	....	—	
Feb. 16 and 17					Listless since 2:30 p.m., at 5:45 p.m. marked hypoglycemic symptoms; 25 g glucose given, followed by meal containing 100 g available glucose. At 10:40 p.m. hypoglycemic reaction, blood sugar 48 mg%. At 11:00 p.m. 35 g glucose given, patient asleep. At 8:00 a.m. breakfast containing about 70 g available glucose.
2:30 p.m.- 9:30 p.m.	0	....	....	—	
9:30 '' - 4:00 a.m.	0	....	....	—	
4:00 a.m.- 8:00 ''	0	24	13	++	
8:00 '' -10:00 ''	0	13	4.6	++	
Feb. 26 and 27					At 11:30 a.m., just before lunch, hypoglycemic reaction. Lunch given, containing 100 g available glucose. Patient listless, asleep 2:00 p.m.
10:30 p.m.- 8:30 a.m.	0	....	....	—	
8:30 a.m.- 2:00 p.m.	0	....	....	+	
2:00 p.m.- 4:30 ''	0	420	546	++++	

A more illustrative case, a young woman with a serious derangement in her carbohydrate metabolism, has been under our continual observation during the last 2 years. Her blood sugar frequently dropped to 30 mg % and even lower, occasionally causing unconsciousness, at variable hours of the day or night. At a time when this condition confined her to the hospital for several months, we found that hypoglycemic periods were often accompanied by ketonuria. In view of the fact that she received 300 g of carbohydrates daily in her food, and in addition to this large amounts of glucose during hypoglycemic attacks, the ketonuria was altogether inconsistent with the current concepts of ketogenesis, antiketogenesis and ketolysis.

Of numerous instances of hypoglycemia accompanied by ketonuria in this case, 3 examples are presented in Table II; the only reason for selecting these particular periods is that ketonuria during these is demonstrated not only by the customary qualitative test but also by quantitative estimation. It is evident from our data, without much comment, that protracted hypoglycemic conditions may persist and can entail ketonuria despite carbohydrate intake that is far beyond the measure normally consumed.

When hypoglycemia persists during the administration of glucose, practically all of the glucose is deposited as glycogen in the peripheral tissues, while at the same time glycogen breakdown in the liver continues at an increased rate. When hepatic glycogenolysis is accelerated, the formation of ketone bodies is also increased.[1] The observations on February 3 and 4 clearly show this relationship. It may be noted that ketonuria ceased only after the hypoglycemic attack had subsided; as indicated by a slight glycosuria at the end, the large amounts of injected glucose eventually led to hyperglycemia, and at the same time the qualitative test for acetone became negative. The third period (February 26 and 27) in Table II is noteworthy in this respect, that it shows that ketonuria due to hypoglycemia may develop to serious dimensions. The excretion of ketone bodies rose to more than 0.5 g per hour due to the fact that in this instance hypoglycemia was not combatted as vigorously as in the preceding 2 periods.

Summary. Hypoglycemia, either induced by hyperglycemia or occurring spontaneously ("hyperinsulinism"?), is frequently accompanied by ketosis (ketonemia and ketonuria). Increased rates of formation of ketone acids, known to occur in the liver, are coincident with increased rates of hepatic glycogenolysis.

11781

Effect of Sulfanilamide on Wound Healing.*

MAX TAFFEL AND SAMUEL C. HARVEY.

From the Department of Surgery, Yale University School of Medicine, New Haven, Conn.

Bricker and Graham[1] reported that sulfanilamide had an inhibitory effect upon the healing of stomach wounds in dogs. Their deter-

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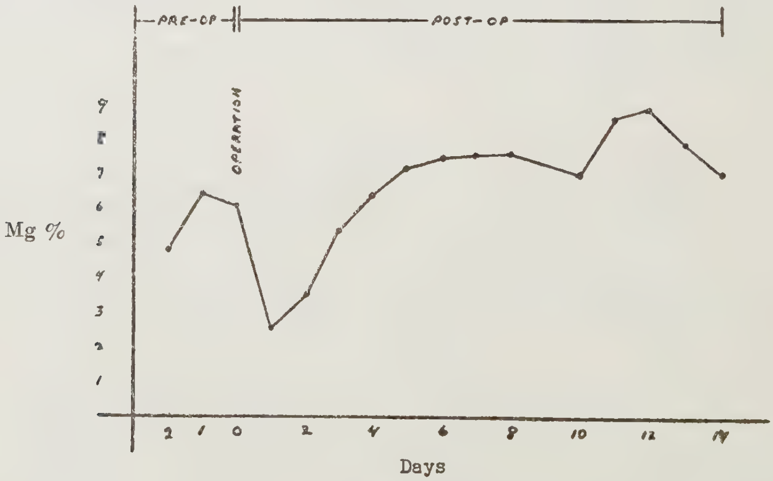
*Aided by a grant from the Fluid Research Funds of Yale University School of Medicine.

[1] Bricker, E. M., and Graham, E. A., *J. A. M. A.*, 1939, **112**, 2593.

minations were made on the 3rd, 5th and 7th postoperative days. In view of the widespread and growing use of sulfanilamide and its related compounds, it seemed important to repeat these studies and to carry the determinations through all the normal phases of healing.

The experiments were performed on adult white rats weighing about 250 g. The tensile strength of the wound was used as an index of healing. In addition comparative histological studies were made of the wounds.

I. Control Group—51 animals. This group was maintained throughout the duration of the experiment on a diet of Purina dog chow. On the 3rd day of the diet a longitudinal wound measuring about one cm in length was made under ether anesthesia through the anterior wall of the stomach at its cardiac end. The wound edges were immediately re-approximated in one layer with a running continuous Connell suture of No. 000 plain catgut which, as has been previously shown, loses its tensile strength well within the 4th day. The abdominal wall was closed with 2 layers of fine No. A silk. On each of the 4th, 6th, 8th, 10th, 12th and 14th postoperative days 5 to 8 animals were sacrificed, and the strength of the wounds immediately determined by distending the stomach with air and noting its bursting point. The details of this method were described by Harvey and Howes.[2] Inasmuch as it has been shown that for approximately 4 days after the injury the wound has only the strength contributed by the holding power of the sutures, no studies were made during this



GRAPH I.

Mean of daily concentrations of free sulfanilamide in blood in all animals of experimental group.

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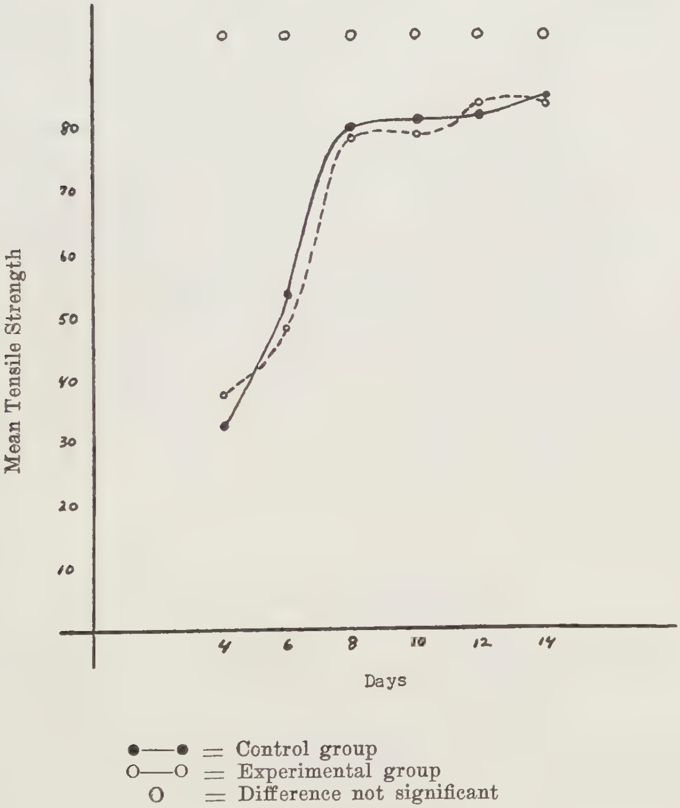
[2] Harvey, S. C., and Howes, E. L., *Ann. Surg.*, 1930, **91**, 641.

first phase of healing. At each time interval the stomachs of 2 animals were allowed to remain intact and were reserved for histological study of the wounds.

II. Experimental Group—48 animals. This group was maintained on a diet of Purina dog chow containing 1% of sulfanilamide[†] by weight.[3, 4] The drug and the chow were finely powdered and both were allowed to mix intimately in a ball mill over many hours. The animals appeared to relish the drug diet and partook of it at once and freely. The average daily consumption was about 20 g per rat. Preliminary studies disclosed that the concentration of free sulfanilamide in the blood was adequate and sustained over a 24-hour period.



GRAPH II.

[†] The sulfanilamide was kindly furnished by Mallinckrodt Chemical Works.

[3] Bieter, R. N., Larson, W. P., Cranston, E. M., and Levine, M., *J. Pharm. and Exp. Therap.* (Proc.), 1939, **66**, 3.

[4] Litchfield, J. T., Jr., White, H. J., and Marshall, E. K., Jr., *J. Pharm. and Exp. Therap.*, 1939, **67**, 437.

In these experiments a determination of the drug concentration in the blood was with few exceptions made once every day on every animal. (Graph 1.) The method of analysis described by Marshall and Cutting[5] was employed. On the 3rd day of the drug diet, wounds exactly similar to those of the control group were made. Tensile strengths and histological studies were similarly carried out from the 4th through the 14th postoperative day. A few of the animals in this group failed for one reason or another to build up and sustain "therapeutic" blood levels of the drug. These were discarded.

In 4 instances, 2 in the control and 2 in the experimental group, small localized abscesses were discovered adjacent to the operative site in the stomach. These were discarded. In the remaining animals the wounds healed *per primam*. In each group the averages of the breaking strength for every postoperative interval, as well as the standard deviation, were computed. Fisher's formula for small samples was applied to determine whether the difference in the means of the 2 groups was statistically significant.

Results. Sulfanilamide did not appear to retard or inhibit the healing of stomach wounds in rats. The curves of healing of these wounds as measured by their tensile strengths were practically identical in the control and experimental groups. (Graph II.) Histological studies of the wounds at each time interval revealed no striking differences in the 2 groups. The oral method of administering the drug was found to be effective and easy of application.

11782 P

Single Responses of Motor Units in Consequence of Volitional Effort.

A. S. Gilson and W. B. Mills.

From the Department of Physiology, Washington University School of Medicine, St. Louis.

Since the demonstration by Adrian and Bronk[1] that action potentials could be recorded from single skeletal motor units* during voluntary muscular activity, several workers have published studies which have made use of this technic. The published material with which we are familiar has, however, dealt with more or less sustained contractions of the muscles voluntarily activated so that records of single motor units have, in all such cases shown repeated discharges. Stetson and Bouman[2] recorded muscle action potentials by means of electrodes placed against the skin. Their records, therefore, were led from many motor units. However, they found that when a quick, ballistic type of movement was made by the subject, the resulting record often revealed one or more groups of discharges, each of these having a duration of about 50 msec. There was, therefore, the possibility that, where a single unit burst appeared, it represented the activity of a considerable number of motor units discharging asynchronously over a period of about 50 msec, but with each motor unit discharging only once.

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[5] Marshall, E. K., Jr., and Cutting, W. C., *Bull. Johns Hopkins Hosp.*, 1938, **63**, 328.

[1] Adrian, E. D., and Bronk, D. W., *J. Physiol.*, 1928, **66**, 81.

* Stetson and Bouman used the term *motor unit* to denote (p. 210) " . . . a time interval during which occur contractions more or less synchronous of groups

It seemed of interest to attempt the registration of a single motor unit discharge resulting from a single, quick, volitional effort. At the present time, 6 normal humans have been used. From each of these there have been obtained records of single discharges of single motor units from one or more of 11 muscles. A high degree of cooperation is required of the subject in order that the muscle unit near the recording electrodes may be made to respond with each

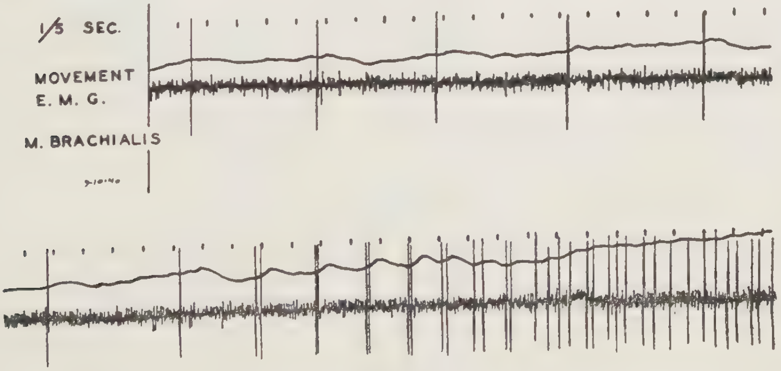

Fig. 1.

Tracing from record showing mechanical movement and electromyogram of brachialis muscle obtained during repeated slight voluntary flexion movements of the forearm. The record is continuous. Toward the end of the record a sustained contraction was produced.

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of fibers . . .'' Liddell and Sherrington[3] employed the term to designate '' . . .the motoneurone-axone and its adjunct muscle fibres . . .'' It is in this latter and more generally accepted sense that the term is employed in this paper.

[2] Stetson, R. H., and Bouman, H. D., *Arch. Neerl. d. Physiol.*, 1935, **20**, 177.

[3] Liddell, E. G. T., and Sherrington, C. S., *Proc. Roy. Soc.*, 1925, **97**B, 511.

volitional effort but without the complicating responses of other units.

A complete discussion of functional and technical aspects of the experiments will not be undertaken here but Fig. 1 indicates the type of record which has been obtained. The record has been traced in ink to facilitate reproduction. Two steel sewing needles (No. 12), lacquered to the extreme tips, led to the grids of a differential amplifier. An unlacquered needle was grounded. The 3 needles were inserted close together in the brachialis muscle. The heel of the hand rested on the diaphragm of a tambour and served to record a slight flexor movement of the forearm. During the first part of the record, discrete volitional efforts at intervals of about 0.8 sec each resulted in a single discharge of the single motor neurone which innervated the group of muscle fibers close to a recording electrode. Other motor neurones may or may not have sent impulses to other muscle fibers less favorably situated for recording. Somewhat quicker movements caused the appearance of double spikes due either to repetitive discharge of the original unit or to the introduction of potentials from a second nearby motor unit which had previously been inactive. With sustained effort the usual rhythmic discharge is recorded.

It has been generally believed that the simplest volitional movement possible involves a brief but rhythmic discharge of impulses from the anterior horn cells concerned. The present studies indicate that when the volitional movement is sufficiently slight and brief, the motor neurones may discharge only once.

11783

Retention of Orally Administered Radio-Phosphorus by Mice.*

LOWELL A. ERF, L. W. TUTTLE AND K. G. SCOTT. (Introduced by J. H. Lawrence.)

From Crocker Radiation Laboratory, University of California, Berkeley, California.

This experiment was devised to determine if the whole body retention of orally administered radio-phosphorus could be increased or decreased by the simultaneous administration of various foods or

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* Acknowledgment is made to the John and Mary R. Markle Foundation for financial assistance and to the staff of the Radiation Laboratory for their generous coöperation in supplying radio-phosphorus to carry out the experiment.

salts. By increasing the amount of radio-phosphorus absorbed, less would be required orally to build up a given concentration in tissues of patients suffering with various generalized neoplastic and near-neoplastic diseases and to whom radioactive agents are given in therapeutic attempts.

Methods and Materials. Three groups of closely related Swiss mice which totaled 190 in number and which were of 30, 70 and 250 days of age respectively, and an additional group of 64 highly inbred mice of the C.B.A. strain, which were 120 days old, were used. All of the 254 mice were placed in cages with wire floors to prevent coprophagy. The males and females were equally divided in each experiment and separated.

Phosphorus. The radioactive phosphorus was produced for us by the Lawrence and Cooksey[1] cyclotron and was converted into neutral isotonic sodium phosphate. 0.25 cc containing 3.7 mg of sodium phosphate and emitting beta radiation equivalent to 5 microcuries,[†] when administered, was given by stomach tube to each of the Swiss mice, and 0.5 cc containing 7.5 mg Na_2HPO_4 and emitting 2.86 microcuries was given to each of the C.B.A. mice. A small and undetermined amount of naturally occurring non-radioactive phosphorus was present in the food supplements, which were simultaneously administered.

Diet. The regular diet consisted of Purina dog chow *ad lib.,* oats and lettuce. This was supplied to all the mice previous to the fasting periods of 20-24 hours and immediately after the administration of radio-phosphorus and the accompanying foods and salts. The carbohydrates—glucose which requires phosphorylation for absorption and mannose that does not—were given in solutions of 10% concentration which are optimum for maximum speed of absorption.[2] Glycine, which is a simple amino acid, was administered in a solution of 10% concentration and raw egg albumen of 50% concentration. The fats—saturated lauric acid, unsaturated oleic acid and a good quality of fortified cod liver oil containing vitamins A, B, D and G—were administered in 0.5 cc doses. See table for concentrations. Ferric and ferrous sulfate were given in doses comparable with those prescribed for human beings. From 6 fasting female mice of one group, 1 cc of blood was withdrawn from the tails just prior to the administration of radio-phosphorus and glucose.

All of the animals were sacrificed at the end of a 72-hour period,

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[1] Lawrence, E. O., and Cooksey, D., *Phys. Rev.*, 1936, **50**, 1131.

[†] Uranium standard.

[2] Verzar, F., *Absorption from the Intestines,* Longmans, Green and Company, 1936.

TABLE I.

	No. of mice	Age (days)	Avg g wt	Amt and type of food or agent administered simultaneously with the radio-phosphorus per mouse	Amt of P^{32} (μ/mouse) retained at end of 3-day period‡	Avg % of retention per mouse	% of retention per g of mouse
Swiss*							
Young	20	30	16.1	.25 cc H_2O	1.30	26	1.6
	19	30	16.8	*Ad lib.*, regular diet	1.44	28.8	1.71
	17	30	16.7	.25 cc 10% sol. of glucose	1.25	25.0	1.5
	19	30	15.6	.25 cc aq. sol. ferrous sulfate (10 mg)	.71	14.2	.91
Adult	20	70	23.4	As above (water)	1.03	20.6	.88
	20	70	22.6	” ” (regular diet)	1.35	27.0	1.19
	16	70	20.7	” ” (glucose)	1.37	27.4	1.32
	18	70	21.6	” ” (ferrous sulfate)	.61	12.2	.57
Old adult	10	250	29.0	” ” (water)	1.45	29.0	1.00
	10	250	31.2	” ” (regular diet)	1.59	31.8	1.02
	9	250	31.9	” ” (glucose)	2.04	40.7	1.28
	6	250	31.9	” ” (ferrous sulfate)	.54	10.8	.34
1 cc of blood was withdrawn from the tails of the 6 females below.							
	6	70	19.2	.5 cc of 10 % sol. of glucose	1.53	30.6	1.58
Total	190						
C.B.A.†							
Adult	10	120	19.7	.5 cc water	.97	34.0	1.72
	10	120	21.5	.5 ” of 10% sol. glucose	1.07	37.4	1.72
	10	120	20.0	.5 ” of 10% sol. glucose	1.24	43.5	2.18
	5	120	22.6	.5 ” of 10% sol. mannose	.97	34.0	1.50
	5	120	22.2	.5 ” of 50% sol. egg white	.96	33.6	1.51
	5	120	20.6	.5 ” cod liver oil	1.07	37.4	1.81
	5	120	21.6	.5 cc aq. susp. lauric acid (35 mg)	.87	30.4	1.40
	5	120	20.5	.5 cc aq. susp. oleic acid (100 mg)	1.13	39.6	1.93
	5	120	16.5	.5 cc aq. sol. ferrous sulfate (10 mg)	.51	17.8	1.08
	4	120	18.2	.5 cc aq. sol. ferric sulfate (10 mg)	.73	25.5	1.41
Total	64						
Grand Total	254						

SUMMARY.

Weighted avg % per g retention of P^{32} disregarding age.

	No. of mice	Agent used	%
	60	Water	1.26
	49	Regular diet	1.30
	52	Glucose	1.71
	48	Ferrous sulfate	.82
Total	209		

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*Each of the Swiss mice received 5 microcuries of radiophosphorus in .25 cc of isotonic Na_2HPO_4 by stomach tube.

†Each of the C.B.A. mice received 2.86 microcuries of radiophosphorus in .5 cc of isotonic Na_2HPO_4 by stomach tube. All mice were fasted 24 hours before administration of radiophosphorus except those Swiss mice to whom regular diet was given.

‡Corrected for decay of radiophosphorus.

since it was assumed that all of the unabsorbed radio-phosphorus would have been excreted from the gastro-intestinal tract during that interval of time. The bodies of the mice in each experiment were pooled and placed in large crucibles and ashed at 520°C. The ash was then thoroughly mixed and an aliquot portion assayed for radioactivity. The results of the assay were corrected for the rate of decay of the radioactive phosphorus, the half-life of which is 14.3 days, to the date of administration.

Results. (See Table I.) The results are discussed in relation to the strain of mice used, since the rate of metabolism (presumably) differs in the 2 strains.

a. The 190 Swiss mice were divided into 3 groups—young, adult and old adult. The control animals, those that received labelled phosphorus alone, retained respectively 1.6%, 0.88% and 1.00% per gram of body weight of the radio-phosphorus administered, at the end of the third day; those maintained on a regular diet 1.71%, 1.19% and 1.02% respectively; those given glucose simultaneously with the radio-phosphorus 1.5%, 1.32% and 1.28% respectively, while those to whom ferrous sulfate was administered retained only 0.91%, 0.57% and 0.34% respectively. It is apparent that the administration of glucose with the labelled phosphorus tends to increase the absorption[3] while ferrous sulfate markedly decreases it. The results indicate that the young mice tend to retain more of the radio-phosphorus per gram of whole body than the older groups.

b. The 64 C.B.A. mice were divided into 10 groups of varying numbers. Only in those groups of mice receiving glucose and unsaturated oleic acid, as supplements, were there significant increases in retention above the control group. The iron salts again were associated with decreased retention.

The weighted mean value for the retention at the end of 3 days by all 60 of the control mice who received no supplement was 1.26% of the phosphorus administered, per gram of whole body. The mean retention by the 52 mice who received glucose supplement was 1.71% per gram while the 49 mice on regular diet retained 1.30% per gram. For the 48 mice receiving ferrous sulfate, the mean retention was 0.82% per gram.

In the small group of 6 female mice there was an indication that active hematopoiesis tended to increase the retention of radio-phosphorus.

The following experiment was devised to determine the differences

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[3] Laszt, L., Intestinal Sugar Absorption and Phosphate Metabolism. *Nature*, 1940, **145**, 899.

TABLE II.

		No. of mice	received	Avg retention of radiophosphorus in % of dose per g of whole mouse on seventh day after administration
(Each mouse received .006 mg Na_2HPO_4)	A	2	NaCl and P^{32} intraperitoneally	2.85
		2	NaCl and P^{32} by stomach tube	2.1
(Each mouse received 7.5 mg Na_2HPO_4)	B	2	P^{31} and P^{32} intraperitoneally	1.0
		2	P^{31} and P^{32} by stomach tube	1.2

in retention when markedly different quantities of inert phosphorus accompanied the administration of radio-phosphorus.

0.2 cc of a sodium phosphate solution, each cubic centimeter containing 0.3 mg sodium phosphate and emitting beta radiation equivalent to 194 microcuries, was placed in 10 cc of distilled water. This solution was divided equally and to one half .045 g of sodium chloride was added and to the other .075 g of sodium phosphate. 0.5 cc of these isotonic solutions were given either by stomach tube or intraperitoneally to 8 closely related C.B.A. mice of same age and weight. Each mouse received 1.91 microcuries of radiation on the date of administration, but 4 (A) received 1250 times more phosphorus than the remaining 4 (B). Seven days later the bodies of the animals were assayed and the results corrected for decay of radioactive phosphorus. (Table II.)

Thus it is observed that the retention of radiophosphorus varies to only a limited extent when different amounts of inert phosphorus accompany it on administration.

In explanation of the results, it would seem that absorption of phosphorus takes place most readily in the upper regions of intestines because the glucose which is readily absorbed as a phosphorylated compound is associated with an increased retention when compared with the retention by mice fed food of the regular diet, which requires longer periods of time for assimilation and absorption. Radio-phosphorus may be absorbed by at least 2 possible mechanisms: (1) diffusion as inorganic phosphate, or (2) by phosphorylation with glucose, etc., present in the gastro-intestinal tract. Therefore, when glucose is administered simultaneously with phosphorus, the first and second mechanisms are operative, while, when phosphorus is administered alone, only the first is operative. There is some slight evidence in the results obtained from the C.B.A.

groups of mice that cod liver oil and oleic acid play rôles similar to glucose.

In the oral administration of radio-phosphorus, in therapeutic attempts, it would seem desirable to administer the P^{32} on an empty stomach and with a 10% solution of glucose in order to obtain greater absorption and retention. The simultaneous administration of iron salts should be avoided.

The greater retention of P^{32} in the young animals can be explained partially on the basis of a more rapid growth, particularly of the osseous system.

Summary. Glucose increased and iron salts decreased the absorption of radioactive phosphorus from the gastro-intestinal tract.

11784 P

Choline, Creatine and the "Labile Methyl" Supply.

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Hemorrhagic degeneration, characterized by renal hemorrhage and fatty livers, occurs in young rats on diets low in choline and in methylated compounds possessing choline-like activity.[1] Supplements of choline,[1] methionine[1] or betaine[2] readily prevent this dietary deficiency. The effect of creatine in decreasing the occurrence of renal lesions is shown in Table I. The liver fat is not significantly affected unless both creatine and choline are fed. Prevention of the fatty liver requires more choline than is necessary for the prevention of renal hemorrhage.[1] The experiments in Table I demonstrate that supplements of creatine decrease the severity of but do not prevent hemorrhagic degeneration. This effect differs from that of methionine and betaine in that only partial protection occurs and the degree of protection is not increased by larger supplements of creatine.

The following explanation, which deals with the transfer of essential methyl groups and not with the synthesis of the rest of a

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[1] Griffith, W. H., and Wade, N. J., *J. Biol. Chem.*, 1939, **131**, 567; 1940, **132**, 627.

[2] Griffith, W. H., and Mulford, D. J., unpublished experiments reported to the Division of Biological Chemistry of the American Chemical Society in Detroit, Sept. 9 to 13, 1940.

TABLE I.

Effect of Creatine on Severity of Hemorrhagic Degeneration in Young Male Rats, 38 to 42 g in Weight and 21 to 26 Days of Age, During an 8-day Experimental Period.

	Supplement mg per g of food	No. of rats	Final body wt, g	Kidney wt, % of body wt	Liver fat, % of liver	Renal lesions %
Basal AC 50*		88	50	1.94	19.9	95
+ Creatine	2	42	58	1.43	20.6	74
+ ”	5	58	57	1.42	17.5	67
+ ”	10	21	61	1.41	18.0	67
+ Choline Chloride	0.4	56	61	1.05	12.5	4
+ Creatine and Choline	5 0.4	21	61	1.00	7.7	0

*Basal diet consisted of casein—18, lard—20, sucrose—48.9, salt mixture—4, calcium carbonate—1, yeast—6, fortified fish liver oil (Natola)—0.1, and agar—2. Average food intake—4.5 to 5.5 g per day.

methylated molecule, is suggested for these results. An adequate supply of compounds containing labile methyl groups is indispensable in the diet of young rats. The occurrence of hemorrhagic degeneration is evidence of a deficiency of choline *and of the labile methyl supply*. Supplements of choline, methionine and betaine contribute to this reserve. On the other hand, the methyl group of creatine is not available for the synthesis of choline. However, dietary creatine does spare the labile methyl supply and makes available for choline formation those methyl groups which otherwise would be used in the synthesis of required creatine.

Choline may or may not be directly involved in all of the various manifestations of hemorrhagic degeneration. It may be required as such for instance in the formation of choline phospholipids or of acetyl choline and it may function indirectly by increasing the labile methyl supply which is required for methylations in metabolism.[3–6] Creatine, unlike choline, apparently does not supplement the stores of available methyl in the body.

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[3] du Vigneaud, V., Chandler, J. B., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, 1939, **131**, 57.

[4] du Vigneaud, V., Chandler, J. B., Cohen, M., and Brown, G. B., *J. Biol. Chem.*, 1940, **134**, 787.

[5] Chandler, J. P., and du Vigneaud, V., *J. Biol. Chem.*, 1940, **135**, 223.

[6] Lewis, H. B., Schultz, J., and Gortner, R. A., Jr., *J. Pharm. and Exp. Ther.*, 1940, **68**, 292.

11785 P

Implantation of Hormonal Substances in the Anterior Chamber of the Eye of Rats.*

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Knowledge of the successful transplantation of endometrium,[1] ovary,[2] anterior pituitary,[3] testis,[4] and adrenal[5] to the anterior chamber of the eye, and the report that excellent results are obtained by subcutaneous implantation of estrogens[6] suggested the possibility that actual hormonal substances implanted in the anterior chamber of the eye might produce characteristic effects. To date ocular implants in rats have been made with testosterone propionate, stilbestrol, progesterone, and pregnant mare serum (PMS).† Clear-cut results have been obtained with all of these substances.

Pellets of testosterone propionate weighing from 1-4.5 mg were introduced into the anterior chamber of the eye of 3 newly castrated, 2 newly hypophysectomized, and 1 long-time hypophysectomized male rats. At autopsy, 21-35 days later, none of the implanted material was discernible in the eye of 2 of the castrates and of 1 of the animals hypophysectomized at the time of implantation. Approximately half of the original weight of substance was recovered from the eye of 1 of the castrates, 1 of the newly hypophysectomized, and of the long-time hypophysectomized male rats. In all 3 of these animals the accessory organs were well stimulated, and in the animal hypophysectomized at the time of implantation the testes were not normal in size but spermatogenesis had been maintained in some tubules.

Stilbestrol pellets weighing about 3 mg were placed in the eyes of

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* Since this article was submitted in June a report by Markee has appeared in the course of which are described some effects of crystalline estrone implants in the eye of monkeys (Markee, J. E., *Contrib. to Embryology*, 1940, **28**, 291).

[1] Markee, J. E., *Am. J. Physiol.*, 1932, **100**, 32.

[2] Goodman, L., *Anat. Rec.*, 1934, **59**, 223.

[3] Haterius, H. O., Schweizer, M., and Charipper, H. A., *Endocrinology*, 1935, **19**, 673.

[4] Turner, C. D., *Am. J. Anat.*, 1938, **63**, 101.

[5] Turner, C. D., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 133.

[6] Deanesly, Ruth, and Parkes, A. S., *Proc. Roy. Soc. London*, s.B., 1937, **124**, 279.

† I am indebted to Dr. Erwin Schwenk of the Schering Corp. for the testosterone propionate, progesterone, and pregnant mare serum, and to Dr. J. A. Morrell, E. R. Squibb and Sons, for the stilbestrol.

2 female rats which had been hypophysectomized for several months. Vaginal estrus occurred about 24 hours later and continued for 9 days, at which time one of the animals died and one was autopsied.

PMS powder was made into pellets the weight of which ranged from 2.5 to 5.5 mg. A pellet was put in the eye of each of 2 female rats and in the eye of each of 6 male rats, all of which were hypophysectomized at the time of implantation. Continuous vaginal estrus was induced and maintained in the females for 17 days. No hormone was recovered from the eyes of these 2 females at autopsy, nor from the eyes of any of the males. Nevertheless the testes of 3 of the 6 hypophysectomized males were moderately large 3 weeks after hypophysectomy. The maintained testes revealed histological evidence of spermatogenic activity. There was but slight stimulation of the interstitial cells, as determined both by the small size of the accessory organs and by the histological appearance of the interstitial tissue. It will be noted that PMS tended to be only gametokinetic when implanted in the eye. The study of Saunders and Cole[7] suggests that this was a quantitative rather than a qualitative manifestation.

A progesterone pellet weighing 4 mg was implanted in the anterior chamber of the eye of a castrated male rat parabiotically joined with a hypophysectomized male. Gonadotropic hyperactivity of the anterior pituitary of the castrate was prevented by the progesterone, since the testes of the hypophysectomized twin did not remain normal, as has been reported,[8] but regressed.

Summary. Pellets of testosterone propionate, stilbestrol, pregnant mare serum, and progesterone implanted in the anterior chamber of the eye of rats produced effects characteristic of each particular substance.

11786 P

Method for Preventing or Diminishing Peritonitis From Leakage After Intestinal Resection or Perforation.

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The high degree of resistance to infection possessed by the peritoneum is well known. Despite that, the mortality from peritonitis following bowel resection (particularly colon) is very high. This is not due to soiling at operation but to leakage from a defect which subsequently develops in the suture line. Attempts to protect the peritoneum against such peritonitis have been made by the intraperitoneal injection of dead bacteria, amniotic fluid, glucose, etc., but without marked success.

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[7] Saunders, F. J., and Cole, H. H., *Endocrinology*, 1938, **23**, 302.

[8] Cutuly, E., and Cutuly, E. C., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 477.

The following observation suggested a method of avoiding such peritonitis. In a case of perforated diverticulitis of the sigmoid the perforated loop was drawn out of the peritoneal cavity and kept wrapped in normal-saline-moistened gauze on the abdominal wall for 25 days. During this time the perforation closed spontaneously and the loop was then freely washed with normal saline and reposited in the peritoneal cavity. The patient made an uneventful recovery.

If it were true that a loop of bowel which had been kept wrapped in saline-moistened gauze out of the peritoneal cavity on the abdominal wall for several weeks could be safely replaced in the peritoneal cavity after merely washing it freely with normal saline solution, then the anastomotic suture line might be exteriorized, kept under observation until safe and then reposited. If it broke down and leaked, it could not leak into the peritoneal cavity which was already sealed off.

Subsequently where resection and anastomosis was done the anastomosed loops were kept in saline-moistened gauze outside the peritoneal cavity on the abdominal wall. When it was seen that the anastomotic line was safely healed (usually after 8-12 days) the bowel loops were freely washed with normal saline solution and reposited in the peritoneal cavity. Clinically, peritonitis did not develop. In 2 instances a small area of necrosis developed at the suture line and perforation with fecal leakage followed. The leakage was outside the abdominal cavity, however, where peritonitis could not occur. One perforation was closed by suture and 8 days later the bowel was reposited in the peritoneal cavity, and in the other case the perforation closed spontaneously after which the bowel was replaced. In all, 19 patients have been treated by exteriorization of the questionable loop until assurance against leakage was certain and then replacement within the peritoneal cavity without the development of peritonitis.

This method is applicable only in cases where the mesenteric attachments are long enough to allow for exteriorization.

It is also applicable in cases of localized inflammation of the bowel with or without perforation since here also the questionable or already perforated loop may be exteriorized and the contribution to more peritonitis stopped until healing has occurred, after which the loop may be reposited.

Summary. A new method for diminishing peritonitis from leakage after resection or perforation of exteriorizable bowel is presented. It is based on the observation that it is safe to reposit within the peritoneal cavity, bowel which has been exteriorized for several weeks, and on the proposition that peritonitis from leakage cannot occur if the leak (actual or potential) is walled off outside the peritoneal cavity.

11787

A Variable in the Sensitivity to Bone-Conducted Sounds.*

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It is well known that the perceptive apparatus essential to hearing is located in the internal ear which is filled with liquid. It is also known that air vibrations are not effectively conducted into liquid directly on account of the impedance differences in the two media. Weber proposed that the middle ear apparatus had the function of a resistance matching transformer which drove, as it were, the air vibrations into the liquid and in this manner conferred great sensitivity in the reactions of the perceptive apparatus to air-conducted sounds. He believed that the tremble in the innermost ossicle, the stapes, located in the oval window, produced a mass shifting in the labyrinth liquid which could come about only through compensation motions at the second window in the internal ear, the round window. This mass shifting of the liquid is thought to result in transverse vibrations of the membranous partition between the two windows and these transverse vibrations, it is thought, in turn constitute the physical activation essential to audition. Curiously enough, the interpretation is founded on two assumptions which are physical impossibilities and the described displacements, which form the basis for all theories of hearing, have never been experimentally demonstrated.

It was known in the sixteenth century that the internal ear also reacted to bone-conducted vibrations and that these bone vibrations passed into the internal ear directly through the investing bony wall. Inasmuch as the bone vibrations are heard qualitatively, the same as air vibrations, it followed that the mechanical reactions in the internal ear to both sound sources were similar and a correlation was effected in the following manner: The bone vibrations produced expansions or contractions in the investing wall, which resulted in pulsating pressure changes in the enclosed liquid. The pressure changes would tend to force both windows in and out synchronously but would not produce the described mass shifting in the liquid if both were equally movable. Accordingly it was assumed that one window was more fixed than the other and this obviously would produce the required differential pressures and the mechanics for hearing bone-conducted sounds would be the same as for air-conducted sounds. If, now, one window became definitely fixed, then the compensations at the second window would be exaggerated and a greater than normal sensitivity to bone-conducted sounds should result. This condition is called a prolonged bone conduction or an increased bone sensitivity. This explanation appeared to fit the evidence in cases of otosclerosis where the stapes is definitely fixed by a bony ankylosis.

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Pohlman and Kranz devised an electro-magnetic receiver which made possible the quantitative testing of the acuity for hearing bone-conducted sounds. They made the observation that in cases in which the oval window was fixed there was no evidence of the increased bone conduction and that the phenomenon was dependent on the masking effect of adventitious noises on the normal hearing individual but not on the deafened individual. In other words, the experimental evidence opposed the accepted explanation.

The English physicist Wheatstone made an important observation on the sensitivity to bone-conducted sounds. He found that when the stem of a vibrating fork was placed in contact with the top of the head, the sound was heard as if it were located in the head. However, when one external ear canal was blocked with the finger or the canal filled with water, the sound was heard in that ear. Pohlman and Kranz reported their experiments employing a bone telephone as a source and under test conditions. They found that the phenomenon was real and that the increased bone sensitivity under both the conditions of blocking the canal and filling with water was similar and limited to the lower frequency range. This observation has been repeatedly confirmed, seemingly quite independently.

Quite recently von Bekesy has proposed another explanation which seems to account not only for the increased bone sensitivity but also for the frequency limitations. He states that when a bone receiver is applied to the forehead the skull tends to vibrate as a whole to low frequencies. The lower jaw, however, is not integral with the remainder of the bones of the head but is separated from it by 2 movable articulations. As a result the lower jaw tends to lag behind the vibrations of the skull as a whole due to its own inertia. This produces a counterthrust at the region of the ear canal which results in air vibrations, which are more effectively conducted to the internal ear when the external auditory canal is occluded. This explanation, while seemingly obvious, presents a number of objections which need not be discussed here.

Fortunately it is possible to test the validity of von Bekesy's explanation. If lack of integration of the lower jaw with the remainder of the skull is indirectly responsible for the effect, then integration of the mandible with the remainder of the head should suppress both the increased bone sensitivity and its frequency limitations. Tests on cases with the bone receiver applied to the forehead with the teeth separated and with the teeth clenched, however, showed no significant change in the phenomenon.

A second test was made in the following manner: Two bone receivers were connected in series with an audiometer. These receivers were so arranged that they could be made to react in the same or in opposite phase. The two receivers were applied to the upper and the lower teeth respectively, with the bite open. In phase, all lag in the mandible, assumed by von Bekesy, should completely disappear and the phenomenon should be suppressed. With the receivers in opposite phase, the assumed lag in the mandible should be exaggerated and not only should the enhanced bone sensitivity show an appreciable increase, but the frequency limitations in the phenomenon should be definitely altered. There is no need to reproduce audiograms because no significant changes in the increased bone sensitivity due to blocking the canal could be detected under either condition. The von Bekesy explanation is accordingly not confirmed.

It is curious that with all of the directed research on audition, the classical observation made by Wheatstone in 1827 has as yet received no adequate explanation. It may be well to emphasize that no explanation, no matter how adequate it may appear to be, is acceptable until it has been definitely confirmed by experimental methods.

11788

Attempts to Transmit Poliomyelitis Virus to Rodents.*

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Although a variety of animals have in the past been injected with the virus of poliomyelitis with the intent to infect them, only a few species of primates have without question been successfully infected. Most experimental work reported has been done either with *Macacus mulatta, (rhesus), M. irus (cynomolgus)* or chimpanzees. These animals are expensive and as a consequence much important experimental work has been neglected because of the prohibitive cost.

Recently, renewed search for a cheaper, suitable, experimental animal was undertaken by several investigators, among them, Armstrong[1] of the National Institute of Health and ourselves. For the most part rodents have been used in these experiments and our results with 25 strains of virus have been negative with all species tested with the exception of the Antelope ground squirrel which occasionally developed questionable neurologic symptoms but which

TABLE I.
Small Rodent Infection Experiment.

Californian strains			
No. strains injected	Negative animals	Genera injected	Armstrong virus % positive
25*	250	*Sigmodon hispidus eremicus* (Calif. cotton rat)	94
6	25	*Sigmodon hispidus hispidus* (So. Car. cotton rat)	96
5	25	*Microtus* (Meadow mouse)	96
6	15	*Neotoma* (wood rat)	41
5	35	*Peromyscus* (deer mouse)	25
2	9	*Perognathus* (pocket mouse)	20
5	20	*Dipodymus* (kangaroo rat)	0

*Viruses injected.

Monkey passage virus from human cord	9
Human cord virus known to be positive in monkeys	9
Monkey passage virus from human stools	5
,, ,, ,, ,, ,, tonsils	1
,, ,, ,, ,, ,, adenoids	1
Total	25

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Armstrong, Charles, *Pub. Health Rep.*, 1939, **54**, 1719.

we cannot state definitely were caused by poliomyelitis virus. Armstrong,[1] on the other hand, reports successful infection of the cotton rat, *Sigmodon hispidus hispidus,* with a strain of virus from Lansing, Michigan.

Table I gives a summary of recent attempts on our part to infect Sigmodons as well as several other species of rodents, both with Los Angeles strains of poliomyelitis virus and with the Armstrong virus.

It will be seen from the table that: (1) Rodents of the genera listed have been refractory to inoculation with a number of strains of poliomyelitis virus collected in Los Angeles. (2) These same animals are subsequently susceptible to inoculation with the Armstrong virus.

It is known that rodents occasionally harbor natural infectious neurotropic viruses, *e.g.,* lymphocytic choriomeningitis has been isolated from white mice, and Theiler[2, 3] and Olitsky[4, 5] report natural infections in mice of an encephalomyelitis virus, which produces typical paralytic symptoms.

Toomey and Takacs[6] and Jungeblut and Sanders[7] report attempts to infect the cotton rat with strains of poliomyelitis virus. Both record negative results with a number of poliomyelitis strains. Jungeblut and Sanders, however, observed neurotropic symptoms in rodents inoculated with one strain of poliomyelitis virus but this may be interpreted as being due to a virus native to the rodent rather than to a human poliomyelitis virus transmitted to mice.

In the light of such current findings one may raise the question whether the Armstrong (Lansing) virus is a poliomyelitis virus that has become adapted to the rodent or whether it is a rodent virus that has become adapted to the monkey.

At least insofar as practical application to poliomyelitis research is concerned, it appears that adaptation of a poliomyelitis virus to the rodent is so rare that such animals cannot be utilized for routine experimental work in the isolation and comparison of strains of virus.

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[2] Theiler, M., *Science,* 1934, **80**, 122.

[3] Theiler, M., *J. Exp. Med.*, 1937, **65**, 705.

[4] Olitsky, P. K., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 434.

[5] Olitsky, P. K., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 296.

[6] Toomey, John A., and Takacs, Wm. S., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 536.

[7] Jungeblut, Claus W., and Sanders, Murray, PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 375.

11789

Effects of Iodine Therapy on Blood Iodine and Basal Metabolic Rate in Pregnancy.*

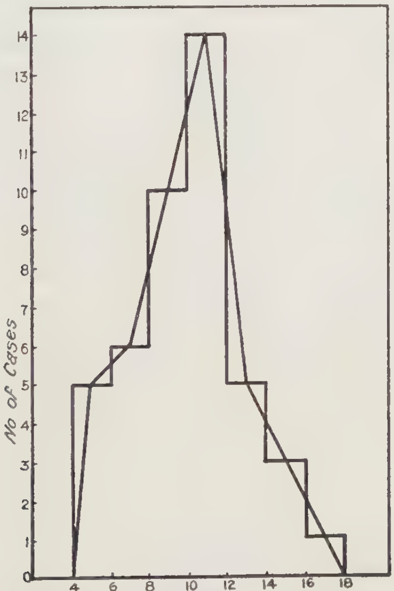
N. M. Phatak, F. B. Zener and N. A. David. (Introduced by E. S. West.)

From the Department of Pharmacology, University of Oregon Medical School, Portland, Oregon.

No definite correlation has been established so far between basal metabolic rate and the advance of pregnancy, but it is generally accepted that the basal metabolism tends to increase in pregnancy. This rise of B.M.R. may well be due to an acceleration of the activity of the thyroid gland in demand for increased iodine requirements during pregnancy. Maurer[1] and Bokelmann and Scheringer[2] have reported increased blood iodine values during the latter part of pregnancy.

For our study, Stevens'[3] method has been used to determine blood

FIG. I



NORMAL BLOOD IODINE DISTRIBUTION
44 CASES

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* Supported by a grant from Ciba Pharmaceutical Company, Summit, N. J.

[1] Maurer, E. E., *Arch. f. Gynak.*, 1927, **130**, 70.

[2] Bokelmann, O., and Scheringer, W., *Arch. f. Gynak.*, 1931, **143**, 512.

[3] Stevens, C. D., *J. Lab. and Clin. Med.*, 1937, **22**, 1074.

TABLE I.
Basal Metabolic Rate and Blood Iodine in Control Group (Untreated Pregnant Women).

	1st trimester	2nd trimester	3rd trimester	At delivery	6 weeks post-partum
No. of cases	14.0	12.0	34.0	27.0	15.0
B.M.R. %	+15.4	+25.7	+27.0	—	+14.1
Blood iodine, γ per 100 cc	17.2	15.0	16.5	20.3	13.6

TABLE II.
Basal Metabolic Rate and Blood Iodine in Treated Group of Pregnant Women

	1st trimester	2nd trimester	3rd trimester	At delivery	6 weeks post-partum
No. of cases	14.0	12.0	13.0	8.0	6.0
B.M.R. %	+15.4	+13.0	+11.0	—	+ 3.0
Blood iodine, γ per 100 cc	17.2	79.2	40.8	27.2	23.6

iodine. The results are shown in Fig. 1. In this group of 44 normal individuals (27 medical students and 17 non-pregnant women), the average blood iodine value lies between 8 to 12 γ per 100 cc.

In Tables I and II are shown the average values for the basal metabolic rates and for blood iodine levels in pregnant individuals, both with and without supplemental iodine therapy.†

It is a common clinical observation that women who do not receive supplemental iodine therapy often suffer from obesity and/or menstrual disorders of various types following pregnancy, which may be ascribed to exhaustion of the endocrine system. Occasionally, the stimulation during pregnancy and labor may be so severe as to cause a toxic hyperthyroiditis. It is also noted that women receiving iodine during pregnancy feel better, are able to nurse their babies well due to an increased milk secretion and regain their antegravid state of well-being much sooner than otherwise.

Conclusions. There is a definite relationship between the advance of pregnancy and the basal metabolism of the gravid individual. The blood iodine level during pregnancy tends to increase. When iodine medication is used during pregnancy, the basal metabolic rate tends to remain close to the normal range of the non-pregnant individual. The supplemental iodine is utilized to a greater extent during the third trimester of pregnancy for at this time the blood iodine level decreases in contrast with the rise seen when iodine medication is withheld.

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† Iodine therapy consisted of Lipoiodine-Ciba, N. N. R. 0.3 g daily all through pregnancy to term.

11790 P

Biotin in Elementary Bodies of Vaccinia.*

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Studies of the constituents of elementary bodies of vaccinia have shown that preparations purified by a standard technic exhibit uniformity in their chemical constitution and in their biological activity.[1, 2] The virus appears exceedingly complex and therefore the view that respiratory catalysts, which are known to play an important rôle in bacterial metabolism, may function in the organization of elementary bodies of vaccinia is not untenable. With this in mind, we have examined elementary bodies for growth-factors.

By means of a technic similar to that described by McDaniel, Woolley, and Peterson,[3] vaccine-virus has been shown to contain relatively large quantities of a substance capable of stimulating the growth of *Clostridium butylicum* in a synthetic medium. Moreover, partial hydrolysis of the virus by normal alkali or 4 normal sulfuric acid gives rise to increased quantities of this substance in active form, which, on the basis of studies similar to those made by Snell, Eakins, and Williams,[4] and others[5, 6] on liver, yeast, egg-yolk, etc., we believe to be biotin.

Two recognized technics exist for the microbiological assay of biotin. The first, employed by Snell, Eakins, and Williams[4] depends upon the growth-stimulus afforded by this substance to a standardized strain of yeast, while the second depends upon the stimulus afforded to the growth of *Clostridium butylicum* in a synthetic medium devoid of biotin.[3] Since the growth-requirements are simpler and somewhat better understood in the case of *Clostridium butylicum,* the second technic was adopted as affording the greatest guarantee against introducing with the material to be tested other physiologically active substances.

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* We wish to acknowledge our indebtedness to Dr. D. W. Woolley for advice in the use of *Clostridium butylicum* in biotin-assay.

[1] Hoagland, C. L., Smadel, J. E., and Rivers, T. M., *J. Exp. Med.*, 1940, **71**, 737.

[2] Hoagland, C. L., Lavin, G. I., Smadel, J. E., and Rivers, T. M., *J. Exp. Med.*, 1940, **72**, 139.

[3] McDaniel, L. E., Woolley, D. W., and Peterson, W. H., *J. Bact.*, 1939, **37**, 259.

[4] Snell, E. E., Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, 1940, **62**, 175.

[5] Weizmann, C., and Rosenfeld, B., *Biochem. J.*, 1937, **31**, 619.

[6] Snell, E. E., and Williams, R. J., *J. Am. Chem. Soc.*, 1939, **61**, 3594.

Elementary bodies of vaccinia were prepared for biotin-assay in 3 ways: first, as dried virus, resuspended by physical agitation; second, by dissolving the dry virus in normal alkali with the aid of heat; and third, by refluxing the dry virus with 4 normal sulfuric acid for 30 minutes at 100°C. The hydrolysates were neutralized with normal acid and alkali, respectively, before they were added to the assay-medium.

A set of standards, consisting of biotin in graded amounts from 0.00004 to 0.0035 γ in a synthetic medium made up according to McDaniel, Woolley, and Peterson[3] which contained 2% dextrose, 0.1% asparagin, and mineral salts, was inoculated with a standard suspension of the vegetative form of *Clostridium butylicum*. The biotin used as a standard was purchased from the S.M.A. Corporation in the form of a concentrate containing 100 γ of active substance per cc when compared with crystalline biotin.

Another set of media in which biotin was replaced by virus-material to be tested was inoculated with a similar suspension of *Clostridium butylicum* and the 2 sets were incubated simultaneously at 37°C for 72 hours in an anaërobic oat jar. All other substances required for the growth of *Clostridium butylicum* were present in excess in the medium so that the degree of growth was proportional to the concentration of biotin added.

Turbidity resulting from the growth of *Clostridium butylicum* was measured by means of an Evelyn-type photocell-colorimeter[7] with the galvanometer scale adjusted to read between 0, when the tube contained media and inoculum only, and 100, when no light was admitted to the photronic cell.

Although elementary bodies alone, with no treatment except autoclaving in the synthetic media prior to inoculation with organisms, yield comparatively large amounts of the growth-factor for *Clostridium butylicum,* partial hydrolysis by normal alkali or 4 normal sulfuric acid yields a ten-fold or even greater increase of this substance. Extraction with alkali and acid serves two purposes: first, to demonstrate resistance of the growth-factor to these substances, and second, to demonstrate an increase in amount after partial hydrolysis. An increased yield of the factor by treatment of the virus with these agents is analogous to the increase in amount of biotin noted in preparations from liver following autolysis.[4]

No absolute value for the concentration of biotin in elementary bodies of vaccinia can be given at this time, but it would appear to approach that observed in egg-yolk, liver, and molasses,[4] which until now have been considered the richest known sources of biotin. Preliminary studies indicate that in the successive steps of purification of virus, a marked concentration of biotin occurs, parallel with the increase in viral activity. Further studies are needed to establish the degree of hydrolysis necessary for complete release of biotin from vaccine-virus.

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[7] Evelyn, K. A., *J. Biol. Chem.*, 1936, **115**, 63.

The fact that a bacterial growth-factor is associated with elementary bodies of vaccinia is important, but definite proof that such a substance represents an integral part of the virus constitutes a larger problem. Studies on the possible rôle of biotin and other growth-catalysts in the metabolism of vaccine-virus are under way, and will be reported in a later communication.

11791

Blood Volume in Cobalt Polycythemia.

John E. Davis.

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Cobalt polycythemia was first produced in rats by Waltner and Waltner.[1] Orten, *et al.*,[2] reported that cobalt polycythemia in the rat was accompanied by a definite increase in blood volume which was due to an increase of cell volume.

We have often produced polycythemia in dogs by feeding cobaltous chloride[3] but have only recently made blood volume determinations on such animals. It is the purpose of the present communication to report the results of these studies.

Procedure. Erythrocyte counts and hemoglobin percentages (Sahli) were determined regularly on 3 dogs which were fed a uniform diet of Purina dog chow. Blood volume was determined by the method of Keith, Rowntree, and Geraghty[4] using the dye brilliant vital red (Evans). Blood samples were drawn from the external saphenous vein while the dogs were lying quietly on a table, at least 15 hours after the previous daily dose of cobalt. Polycythemia was produced by the daily administration of 2 mg of cobalt per kg, in the form of a dilute solution of cobaltous chloride, by stomach tube.

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[1] Waltner, K., and Waltner, K., *Klin. Wochenschr.*, 1929, **8**, 313.

[2] Orten, J. M., Underhill, F. A., Mugrage, E. R., and Lewis, R. C., *J. Biol. Chem.*, 1933, **99**, 457.

[3] Davis, J. E., *Am. J. Physiol.*, 1938, **122**, 397; 1939, **127**, 322; 1940, **129**, 140.

[4] Keith, N. M., Rowntree, L. G., and Geraghty, J. T., *Arch. Int. Med.*, 1915, **16**, 547.

Results. Fig. 1 shows changes in blood volume and blood cell volume of 3 dogs before and after the onset of cobalt polycythemia. The dashed lines indicate varying periods of time which elapsed between the commencement of cobalt administration and the attainment of polycythemia in the different dogs. It will be seen in Fig. 1 that cobalt induced a significant increase of blood volume, and an even more pronounced increase in the volume of cells in the circulation (average increase, about 20%). The average normal control values for blood volume in these dogs ranged from 8.5 to 9.0% of their body weights.

That polycythemia was produced in these dogs is shown in Table

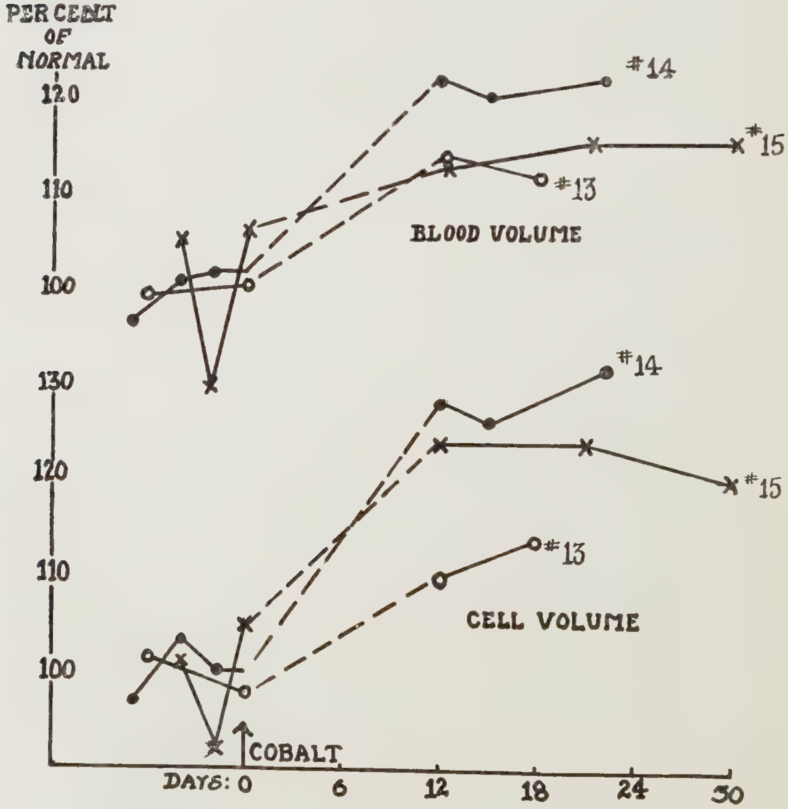

FIG. 1.

Changes in blood volume (above), and blood cell volume (below), in 3 dogs with cobalt polycythemia.

TABLE I.
The Production of Polycythemia by Cobalt in the 3 Dogs Whose Blood Volume Changes are Shown in Fig. 1. Erythrocyte Numbers Are Given in Millions.

	Dog No. 13	Dog No. 14	Dog No. 15
Before Cobalt	5.17	5.78	5.07
	5.26	5.81	5.16
	5.06	5.75	5.28
			5.09
After Cobalt	5.93	6.75	6.29
	6.07	6.84	6.32
	6.22	6.72	6.25
	6.13	6.77	6.30

I, which presents erythrocyte numbers observed before and after the production of cobalt polycythemia. The increases are not great but are significant, ranging from 17 to 22%.

These experiments on dogs confirm, in a qualitative way, the work of Orten, Underhill, Mugrage, and Lewis,[2] which was done on rats.

Conclusion. Cobalt polycythemia in the dog is accompanied by an increase of blood volume which is due chiefly to an increased volume of cells.

11792

Adenomatous Stomach Lesion of the Rat Associated with Heavy *Cysticercus fasciolaris* Infestation.*

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In connection with an extensive compilation of their data on spontaneous tumors of the rat, Bullock and Curtis[1] recorded the interesting observation that a hypertrophic gastritis was frequently found in rats infested with large numbers of *Cysticercus fasciolaris,* the larval form of the cat tapeworm, *Taenia taeniaeformis (crassicollis).* Following the ingestion of the *Taenia* eggs by the rat, the oncospheres localize in the liver, where they lead to the formation of cysts from which the well-known *Cysticercus* sarcomas often develop.[2] The gastritis seems to be definitely associated with the

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* This investigation was aided by a grant from William R. Warner and Co., Inc., New York City.

[1] Bullock, F. D., and Curtis, M. R., *J. Cancer Res.*, 1930, **14**, 1.

[2] Bullock, F. D., and Curtis, M. R., *Proc. New York Path. Soc.*, n.s., 1920, **20**, 149.

heavy infestations produced by massive feedings of *Taenia* eggs.[3] During the course of experiments on feeding *Taenia taeniaeformis* eggs, we also have observed thickened and enlarged stomachs in the rats. In view of the scarcity of experimental approaches to research on tumors of the glandular stomach,[4] it is desirable to call attention to the adenomatous character of the gastric lesions that have been produced in our animals.

Infestations were carried out by feeding portions of terminal proglottids of mature worms or by feeding small pieces of bread on which counted suspensions of eggs had been dropped. Grossly thickened stomachs were observed in all of 7 rats, 3 males and 4 females, which were sacrificed after a heavy *Cysticercus* infestation of 235 to 272 days' duration. In these animals the enlarged livers contained 150 to 200 cysts, displacing most of the liver tissue. An eighth rat, with a less pronounced lesion, had an infestation of 61 cysts for 303 days. These animals included piebald rats of the McCollum strain and albinos of the Wistar and Buffalo strains, all of which were maintained on the McCollum stock diet without supplements. Different animals of the group had been infested at 3 different times by means of *Taenia* worms secured from 3 different cats.

By contrast, in a group of 9 rats of the same strains that were lightly infested, with 5 to 33 cysts of 239 to 330 days' duration, no significant changes were present in the stomachs. The tenth rat of the group had a practically normal stomach, although it had an infestation of 63 liver cysts for 295 days. Likewise, the stomachs of a non-infested group of control rats were normal. In neither the heavily nor lightly infested animals had there yet developed any gross evidence of *Cysticercus* sarcomas at the time of autopsy.

The thickened stomachs varied in size from only slightly larger than normal up to more than twice the normal size. When the animals were sacrificed, the forestomach was usually found contracted and containing little food. The glandular stomach was always practically empty or covered with a thick coating of mucus. As a result of the pronounced thickening, the lumen of the stomach was considerably narrowed, usually leaving scant space for food.

Grossly the lesion appeared to be confined to the glandular part of the stomach; the forestomach was essentially normal. The mucosal surface of the stomach appeared rough, with an opaque pink or white color. Sometimes small superficial hemorrhagic areas were seen. The

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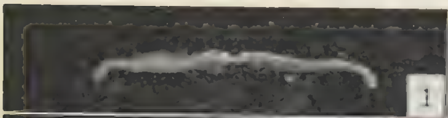
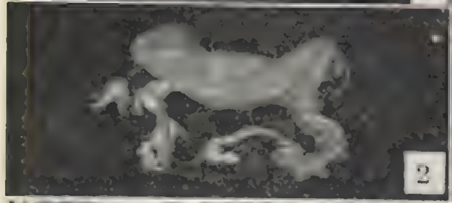
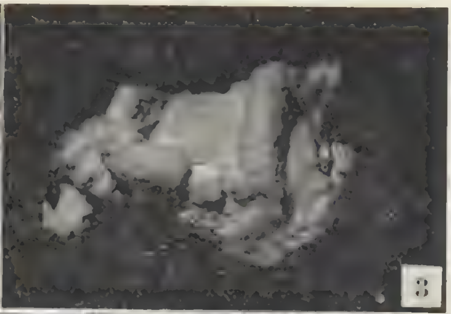
[3] Curtis, M. R., and Dunning, W. F., personal communication.

[4] Klein, A. J., and Palmer, W. L., *Arch. Path.*, 1940, **29**, 814.







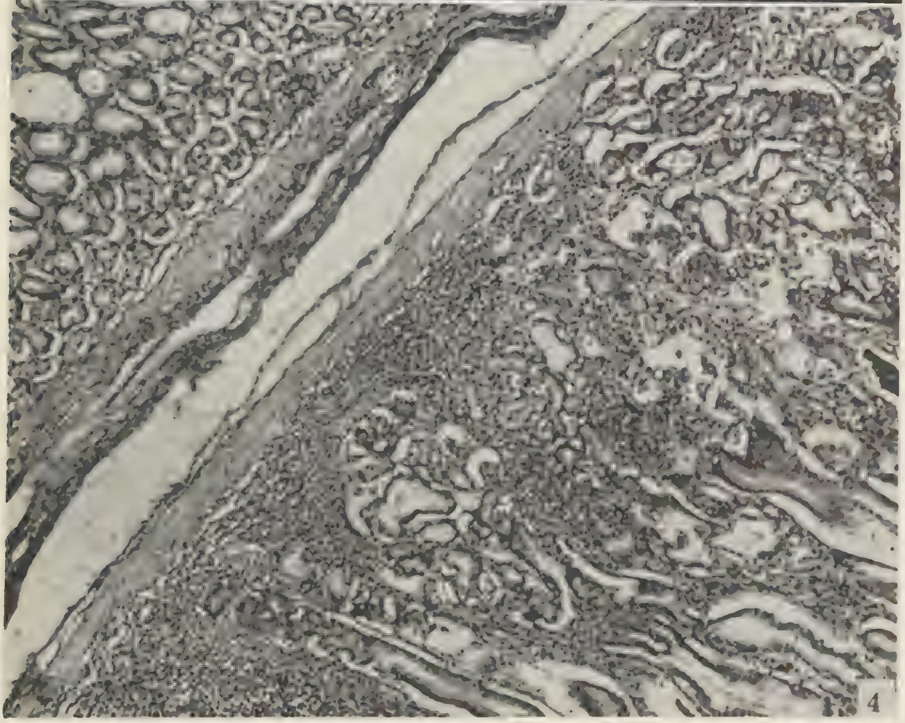


FIG. 1. Cross section of normal glandular stomach of rat. (Actual size.)

FIG. 2. Cross section of adenomatous glandular stomach of rat bearing heavy liver infestation of *Cysticercus*. The stomach has been turned almost inside out to show the size of the 2 large papillary overgrowths, which projected into the lumen. (Actual size.)

FIG. 3. Top view of sectioned stomach shown in Fig. 2. Note the 2 long ridges. (Actual size.)

FIG. 4. Photomicrograph taken near base of right ridge of stomach shown in Fig. 2. Note connective tissue stalk, inflammatory changes in stroma, and abnormal glands. (× 75.)

thickening was in some cases almost general, while in others there were in addition papillary extensions of as much as 7 mm beyond the normal stomach thickness (Fig. 1, 2). The most pronounced part of the overgrowth was in various sites, such as at the border of the forestomach, as symmetrical longitudinal ridges (Fig. 3), or in one case as an enlarged ring around the pylorus.

Microscopically the lesion appeared adenomatous. There was glandular hypertrophy, sometimes arising from large connective tissue stalks (Fig. 4). Many glands appeared abnormal, showing hyperplasia, metaplasia, and necrosis. The stroma showed inflammatory changes in which an extensive eosinophilia was sometimes noted. Enlarged acini were frequently present.

Although the presence of some unrecognized factor, such as another concomitant infection, can not be ruled out, the consistent association of the heavy liver infestation and the adenomatous stomach lesion would seem to indicate that the *Cysticercus* infestation itself is responsible for the adenomatous stomach changes. No gross local parasitic infection has as yet been observed in the routine inspection of the stomachs, or after incubation of the stomachs for one hour at 37°C in physiological saline solutions. The absence of the lesion in lightly infested rats, or in control animals maintained in the same cage with heavily infested rats, is evidence against a contagious infection as the etiological factor. Portions of thickened stomach are now being fed to young rats in attempts to transmit any local infectious agent present.

Andervont and Stewart[5] have reported a spontaneous adenomatous lesion of the stomach in Strain I mice, in which susceptibility to the lesion is inherited. In the case of the adenomatous rat lesion, its production in a piebald strain and in two albino strains apparently eliminates hereditary susceptibility as a major factor in the etiology of the lesion associated with *Cysticercus* infestation.

Bonne and Sandground[6] have reported the experimental production of adenopapillomatous growths in the Javanese monkey by means of *Nochtia nochti*, a nematode living in the stomach. In the case of the *Cysticercus* stomach lesion, it must be noted that the infestation is apparently confined to the liver, thus seeming to eliminate local tissue irritation by the larval worm as a cause of the proliferation. Since the development of the lesion appears to depend upon the presence of a large number of liver cysts, it is likely that the adenomatous change is brought about at a distance from the infestation through the medium of some toxic metabolic product elaborated by the encysted worms. It is also possible that some nutritional alteration takes place as a result of the displacement of the major part of the liver tissue by the cysts. It is interesting that the same organism that often leads to the formation of liver sarcomas in older animals is also associated with the adenomatous gastric lesion which has been described.

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[5] Andervont, H. B., and Stewart, H. L., *Science*, 1937, **86**, 566.

[6] Bonne, C., and Sandground, J. H., *Am. J. Cancer*, 1939, **37**, 173.

Further studies are now in progress on the effect of dietary modifications on the development and possible prevention of the lesion.

Summary. The production of adenomatous stomach lesions of the rat, associated with heavy liver infestations of *Cysticercus fasciolaris,* is reported. The adenomatous stomach lesions have been produced in 3 different strains of rats.

11793 P

A Virus Pneumonia of Syrian Hamsters.*

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In the course of attempts to infect Syrian hamsters (*Cricetus auratus*) 2 to 4 months old, with influenza A, virus strain W. S.,[1] another filterable agent capable of producing a fatal pneumonia in these animals was found. One hamster was inoculated intranasally with mouse-passage influenzal virus (W.S.) and developed slight nasal symptoms. This animal was sacrificed after 8 days and suspensions of lung and turbinate were passed to a second hamster which had large plum-colored pneumonic areas in its lungs when killed at 6 days. Subsequently suspensions of lung and turbinate have been carried through 17 serial intranasal passages in hamsters with the production of pneumonia in each passage except the first and third. The suspensions at each hamster-passage when inoculated intranasally into mice produced a pneumonia which was usually fatal within 6 to 9 days. Attempts to produce a similar pneumonia in hamsters with several other mouse-passage strains of influenzal virus were unsuccessful. We were also unable to repeat the original experiment starting with another mouse-passage of the strain W.S.

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* The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of The Rockefeller Foundation and in coöperation with the California State Department of Public Health.

[1] Taylor, R. M., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 541.

Cultures of lung and turbinate-suspensions on the ordinary bacteriological media gave either no growth or only a few colonies of various kinds of bacteria. A Berkefeld V filtrate of a suspension was infectious for hamsters, but not for mice. The agent failed to pass a Seitz filter.

Signs of infection in the hamster were variable, but usually 2 to 6 days following nasal inoculation the animal sniffed or sneezed and rubbed its nose. Then rhonchi, ruffled fur, dyspnea, and weakness were noted. Death occurred in 6 to 15 days, sometimes preceded by a terminal bloody nasal discharge. The only change in temperature was a terminal fall.

The lungs of dead animals were moist, plum-colored and completely consolidated. The turbinates were swollen and red. Other organs did not appear abnormal. The lungs of animals killed showed lesser degrees of lung-involvement. Some animals died without lung-lesions after having signs of infection. Microscopically there were hyperemia, infiltration and thickening of the alveolar walls with serous and slight cellular exudate in the alveoli. Mononuclear cells predominated. There were extensive areas of partial or complete atelectasis. The bronchi showed little or no abnormality, but evidence of damage was present in the nasal mucosa. No gross or microscopical changes were seen in the brain, heart, liver, spleen, or kidney.

Lung or nasal mucosa was infectious for hamsters and for mice, but no virus was recovered from other organs. Parallel titrations of hamster-material in mice and hamsters indicated that the latter probably were more susceptible.

Subcutaneous, intra-abdominal, or intracerebral inoculation did not result in any sign of infection except, occasionally, sniffing and ruffled fur. Normal hamsters placed in contact, for periods of 1 to 5 days, with animals infected by intranasal inoculation sometimes developed signs of infection and, occasionally, small lung-lesions. Hamsters inoculated subcutaneously, intra-abdominally, and intracerebrally, and also hamsters infected by contact were resistant 3 to 5 weeks later to an intranasal dose of virus that was regularly fatal to control animals. Some of the immune animals had nasal symptoms after reinoculation.

While testing throat-washings by serial intranasal passage of lung-suspensions in mice, we have isolated 3 apparently identical strains of a virus which corresponds in its properties with the mouse-pneumonia virus described by Horsfall and Hahn.[2, 3] Pneumonia-

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[2] Horsfall, F. L., Jr., and Hahn, R. G., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 684.

[3] *Ibid., J. Exp. Med.*, 1940, **71**, 391.

viruses have been isolated from the lungs of normal mice by other workers.[4, 5]

Cross-immunity and neutralization-tests in hamsters and mice indicated that the virus isolated in hamsters was antigenically related to our strain of mouse-pneumonia virus, but was different from influenzal virus. Direct inoculation of mouse-pneumonia virus into hamsters gave the same fatal pneumonia as that following hamster-virus injection. However, the second and third passages in hamsters with the mouse-passage virus gave irregular results.

These agents appear to differ from the pleuropneumonia-like organism described by Edward[6] in that they produce a more rapidly fatal pneumonia of a different type. However, the possibility that a pleuropneumonia-like organism is concerned in the production of hamster-pneumonia cannot be excluded at present.

11794

Electrophoresis of Influenzal A-Virus.

JAQUES BOURDILLON. (Introduced by J. H. Bauer.)

From the Laboratories of the International Health Division, The Rockefeller Foundation, New York.

In a recent publication[1] a study was made of the electrical mobility of the complement-fixing antigen present in mouse-lungs infected with influenzal A-virus.[2] This paper reports a study of the mobility of the virus itself made under the same experimental conditions.

Material and Methods. The material used was a 10% suspension of mouse-lung infected with the PR8 strain, which was dialyzed against various buffers (Table I). It was placed in the medium size cell of the Tiselius apparatus, and the current was allowed to pass for 2 to 3 hours under a potential gradient of about 10 V/cm. It was then sampled at various levels in the cell, and each sample was titrated in mice. Details of the sampling procedure have been given else-

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[4] Dochez, A. R., Mills, K. C., and Milliken, B., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 683.

[5] Gordon, F. B., Freeman, G., and Clampit, J. M., PROC. SOC. EXP. BIOL. AND MED., 1939, **39**, 450.

[6] Edward, Derrick G. F., *J. Path. and Bact.*, 1940, **50**, 409.

[1] Bourdillon, J., and Lennette, E. H., *J. Exp. Med.*, 1940, **72**, 11.

[2] Horsfall, F. L., Jr., Lennette, E. H., Rickard, E. R., Andrewes, C. H., Smith, W., and Stuart-Harris, C. H., *Lancet*, 1940, in press.

TABLE I.
Approximate Electrophoretic Mobility of Influenzal A Virus.

Buffer	(Mobility cm^2 v^{-1} sec^{-1} × 10^5) Ascending	Descending
Phosphate pH 6.8, ionic strength 0.1	—6.5	—4.5
” ” 7.9 ” ” 0.1	—5.5	....
Borate ” 9.8 ” ” 0.04	—8.0	—8.0

TABLE II.
Electrophoresis of Influenza A Virus in Borate Buffer, pH 9.8, Ionic Strength 0.04. Potential Gradient 11.2 V/cm. Time 112 Minutes.

Sample No.	Mean distance of sample from initial ascending boundary (cm)	Virus titer (log)	Antigen titer*
1	14.0	1.0	0
2	11.7	2.3	0
3	9.4	2.7	0
4	7.1	3.7	1:4
5	4.8	5.0+	1:32+
6	2.0	5.0+	1:32+
7	—0.9	5.5+	1:32+
8	—3.2	5.0+	1:32+
9	—5.5	5.5+	1:32+
10	—7.8	4.7	1:16

The compensation which was applied during electrophoresis caused a displacement of the theoretical position of the initial ascending boundary from the middle of the ascending side to the bottom of the descending side.

*Determinations kindly performed by Dr. E. H. Lennette.

where.[1] The virus-titration method used has been described by Horsfall.[3]

Results. Table II gives a simplified account of one experiment. Sample 1 was removed from the bottom of the top section of the cell; samples 2 to 5 from the 2 middle sections on the ascending side; sample 6 from the bottom section; samples 7 to 10 from the 2 middle sections on the descending side. The viral titer was 5.3 log in the original material; it remained about the same in samples 5 to 9. Between samples 5 and 4 there was a drop in titer of at least 1.3 log, or twentyfold; it was paralleled by a drop in antigenic titer of from (probably) 1:64 to 1:4; a further tenfold drop in viral concentration occurred between samples 4 and 3, paralleled by a drop to 0 in antigenic titer; in samples 1 and 2 the viral concentration was only 1/1000 or less that of the original material. Assuming the boundary to be between samples 4 and 5, calculation gave —8×10^{-5} as the most probable value for the mobility of both virus and antigen in this experiment. Similar experiments were made at other pH's, the results of which have been summarized in Table I. In the same buffers, the mobility previously obtained for the complement-fixing antigen was respectively 5.2, 5.4, and 7.8×10^{-5}.[1]

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[3] Horsfall, F. L., Jr., *J. Exp. Med.*, 1939, **70**, 209.

It appears, therefore, that the mobility of the virus and that of the antigen at alkaline pH are, if not identical, at least of the same order of magnitude. Below pH 5.6 the virus was rapidly destroyed so that its mobility could not be investigated in the acid range. The experiments did not suggest that the boundaries were less sharp than those of any protein-fraction studied optically, nor that any considerable endosmotic streaming had taken place, such as was observed by McFarlane[4] with vaccinial virus. It is probable that the absence of important convective currents was due to the concentration-gradient created by the other proteins present in the suspension.

Summary. The electrophoretic mobility of influenzal A-virus has been found to be about -5.5×10^{-5} cm^2 V^{-1} sec^{-1} at pH 7 to 9, ionic strength 0.1; and about -8×10^{-5} at pH 10, ionic strength 0.04. It appears to be the same as that of the complement-fixing antigen.

11795 P

Early Localization of the Thyroid Anlage in *Hyla regilla*.

MIRIAM STOKES. (Introduced by J. Frank Daniel.)
From the Department of Zoology, University of California, Berkeley.

Since the initial work of Vogt[1] on the localization of anlagen in the young amphibian gastrula, investigators have continued to map out on the anuran or urodele gastrula the presumptive areas which in normal development give rise to particular organs or structures. Although Holtfreter[2] has added considerably to the knowledge of these areas by explantation, the major number of the studies have utilized the technique of vital staining.

In a 3mm, tail-bud embryo of the Pacific tree frog, *Hyla regilla*, the thyroid is visible as a ventral outgrowth just anterior to the yolk mass which forms the floor of the archenteron. Röhlich[3] has shown that for *Triton* at least, the major part of the foregut arises from the dorsal lip of the blastopore. The location of the thyroid in the older embryo, however, gives no indication whether its origin is from the dorsal or the ventral lip.

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[4] McFarlane, A. S., *Tr. Faraday Soc.*, 1940, **36**, 257.

[1] Vogt, W., *Arch. f. Entw-mech.*, 1925, **106**, 542.

[2] Holtfreter, J., *Arch. f. Entw-mech.*, 1938, **138**, 522.

[3] Röhlich, K., *Arch. f. Entw-mech.*, 1933, **129**, 794.

Gastrulae were obtained in which the future crescentic blastopore was indicated only by heavier pigmentation or by a slightly puckered appearance of the cells. After removal of the jelly layers, but not the chorion, the embryos were placed in wax-bottomed watch glasses, with the blastopore uppermost. Bits of agar impregnated with Nile blue sulphate according to the method of Stone[4] were placed on the embryo and held firmly in place by a celluloid bridge. After half an hour the agar was removed and the embryo transferred to a dish of pond water. Two days later the animal was either dissected to locate the stain or was fixed by Yntema's method[5] for future sectioning. A total of 40 animals was thus studied, of which 27 were dissected, 8 were sectioned and stained, and 6 were discarded because the stain was too faint for accurate observation.

Results of the study show that the cells which later form the thyroid lie in the median plane and *within* the early blastoporal groove, slightly more toward its ventral lip. The location was clearly demonstrated in 14 embryos. Stain placed on either side of the midline (5 embryos) is found, if at all, only on one side of the thyroid bud. Stain placed dorsal to the groove (5 embryos) later lies in the anterior wall of the foregut, and stain placed ventral to the groove (8 embryos) is pushed caudad by the ventral union of the heart anlagen.

To Vogt's classic map it is therefore possible to add, within the early crescentic blastopore and in the midline of the embryo, the location of the small area which in *Hyla regilla* will give rise to the thyroid gland.

11796

Effect of Low Body Temperature on Toxicity of Drugs.

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Low body temperature is known to produce a stuporous condition in different animals, accompanied by extremely slow respiration and pulse rate, and a decrease in the basal metabolism. Little work has been done on the effect of toxic doses of drugs in animals whose body temperature has been artificially lowered. Dworkin *et al.*[1-3] injected convulsive doses of insulin in cats, woodchucks and dogs previously cooled by means of amytal and ice bath, and found that cooling inhibited convulsions. Koeninck[4] injected strychnine, picrotoxin, muscarine, pilocarpine, caffeine, apomorphine and tetanus toxin into a few *hibernating* bats. All drugs acted slowly, but produced typical symptoms and—except pilocarpine—deaths. Blanchard[5] injected cobra venom and some toxins into hibernating marmots and did not obtain any significant change of toxicity during hibernation. Pfeiffer, Foster, and Slight[6] found in hibernating animals a marked increase in the toxicity of strychnine and caffeine, 10-8 times respectively. Coramine and picrotoxin were twice as toxic, while the lethal doses of cocaine, metrazol and ephedrine remained unchanged; other sympaticomimetic drugs showed a decrease of toxicity in hibernation. It seemed of interest to study the effect of toxic doses of convulsant and other drugs on animals with body temperature artificially lowered by cooling.

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[4] Stone, L. S., *Anat. Rec.*, 1932, **51**, 267.

[5] Yntema, C. L., *J. Exp. Zool.*, 1937, **75**, 75.

Method. Mice were wetted with ice water or 95% alcohol and then placed in front of an electric fan. The body temperature, thus rapidly lowered to 18-20° C, remained between 18 and 22° for the duration of the experiment (rectal temperatures were determined by a thermocouple). The lethal dose of each drug used was first determined on non-cooled mice and this dose was injected into the cooled animals. The mortality ranged in the controls from 33 to 86% (see Table).

TABLE I.
Toxicity of Drugs for Cooled Animals.

Drug*	No. of animals		Survival time		Mortality	
	Cooled	Control	Cooled Min.	Control Min.	Cooled %	Control %
Strychnine sulfate, 3 mg/K	6	7	17	5	100	72
Metrazol, 100 mg/K	6	7	55	5	83	86
Nicotine, 4 mg/K	12	6	24	4	100	33
Amphetamine sulfate, 150 mg/K	6	5	29	16	100	40
Morphine sulfate, 350 mg/K	7	8	105	62	72	62
Cocaine HCl, 200 mg/K	6	6	11	5	100	67
Procaine HCl, 250 mg/K	9	8	17	8	100	63

*All injections for experiments listed in this table were made intraperitoneally.

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[1] Dworkin, S., and Finney, W. H., *Am. J. Physiol.*, 1927, **80**, 75.

[2] Finney, W. H., Dworkin, S., and Cassidy, G. S., *ibid.*, 1927, **80**, 301.

[3] Cassidy, G. S., Dworkin, S., and Finney, W. H., *ibid.*, 1925, **73**, 417.

[4] Koeninck, A., *Arch. f. Physiol.*, 1899, p. 389.

[5] Blanchard, R., *Compt. R. de la soc. de biol.*, 1903, **55**, 734.

[6] Pfeiffer, C., Foster, M. A., and Slight, D., *J. Pharm. and Exp. Therap.*, 1939, **67**, 307.

Results. Cooling depressed convulsions in all animals. The toxicity of the drugs, with the exception of metrazol, was greater in cooled mice than in the corresponding controls, the average total mortality being 95% in the cooled as against 60% in the controls. The slight increase in percentage mortality with morphine is probably not significant. However, the survival time was markedly prolonged in all cooled animals (from about 2 to 11 times). That these changes were independent of the route of administration was indicated by experiments with metrazol and strychnine where intravenous as well as subcutaneous and intraperitoneal injections were made.

Discussion. Our results on toxicity of strychnine and metrazol corroborate those of Pfeiffer *et al.*[6]; however cocaine and amphetamine (benzedrine)* showed in contrast to their effect on hibernating squirrels an increased toxicity in cooled mice. This may be taken as an indication that drugs producing cerebral stimulation are unlike in their effects on hibernating and artificially cooled animals. A further pharmacological analysis may be able to throw some light on similarities and differences in the mechanisms involved in hibernation on one side and artificial cooling on the other.

The prolongation of life in cooled animals may be partly due to delayed resorption of the drug. The general depression of the rate of metabolism probably contributes another important factor, by decreasing the demands of the tissues for vital substances. A similar prolongation of life was observed by one of us in cooled rabbits and cats[7] after lethal doses of insulin. That the depression of convulsions through cooling may be an important mechanism, is suggested by the fact that the most strongly convulsant drugs (strychnine, metrazol, nicotine) show the greatest prolongation of life.

Summary. Artificial cooling of mice resulted in increased mortality after injection of lethal doses of strychnine, nicotine, amphetamine, cocaine, and procaine; no marked difference was observed after metrazol and morphine. Prolongation of surviving time was observed with all animals which succumbed, most markedly after strongly convulsant drugs.

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* Supplied through the courtesy of Smith, Kline and French Laboratories.

[7] Tyler, D. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 278; *ibid.*, 1940, **45**, 117.

11797 P

Some Effects of Physical Fatigue on Peripheral Circulation in Athletes.

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This investigation was prompted by suggestions resulting from observations on the high incidence of recurrent attacks of malaria and predisposition to upper respiratory infections following fatigue. The possible suggestion arose that there might be an effect upon the peripheral circulation to which a clue could be obtained by a study of the blood picture. An earlier report by Hastings on the physiology of fatigue[1] called attention to the fact that following long continued exercise the resistance of erythrocytes to laking increased "at a diminishing rate," but there are no conclusions regarding the condition or proportion of white cells in his study. It was decided to carry on this study on subjects who had exercised until they were greatly fatigued and compare the results of study of the blood picture during this period with the normal blood picture before exercise and with that following recovery from fatigue.

Microscopic studies were made on blood smears, stained with Wright's stain, at intervals before and during the recovery period following fatigue as follows: preliminary examination before exercise, immediately after exercise, and at intervals of one-half hour, one hour and 2 hours after exercise. Forty male volunteers from the various athletic teams and classes were used in this study. A study of the results of the examination of these slides has been tabulated in Table I.

TABLE I.
Relative Change in the Proportion of Cells on the Basis of a Count of 100 White Cells.

	Type of cell							
	Polymorphs		Large lymphocytes		Small lymphocytes		Total lymphocytes	
Results	No. of Cases	%	No. of Cases	%	No. of Cases	%	No. of Cases	%
Relative significant increase (5% or greater)	2	5	26	65	12	30	28	70
No significant change (less than 5%)	12	30	10	25	24	60	9	23
Relative significant decrease (5% or greater)	26	65	4	10	4	10	3	7

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[1] Hastings, Albert B., *U. S. Public Health Bulletin* 117, May, 1931.

The distribution of monocytes, eosinophiles, and basophiles did not vary significantly.

Discussion. From the study of the tabulations it appears that, contrary to expectations, there was an immediate decrease in the relative number of polymorphs in more than one-half of the cases studied immediately after exercise or at the onset of the fatigue phase. Among the lymphocytes the greatest change appears as a relative increase in numbers of large lymphocytes as well as a significant relative increase in the total lymphocytes in more than one-half of the cases studied.

It appears that at the onset of the fatigue phase there is a significant lymphocytosis suggestive of an "irritation phenomenon" such as is frequently found in infections or other depleting conditions.

11798

Location of Inhibitory Respiratory Center in Cerebral Cortex of the Dog.

PERCIVAL BAILEY AND WALTER HAYNES. (Introduced by Arthur Weil.)

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There is considerable discrepancy in the literature concerning the location of the inhibitory respiratory center in the cerebral cortex of the dog. Spencer[1] originally located it in the allocortex, medial to the sulcus rhinalis. Bucy and Case[2] located it just below the anterior extremity of the coronal sulcus and Smith[3] farther posteriorly in the gyrus compositus anterior. Because of this uncertainty we have repeated these experiments using the technic of Bailey and Sweet[4] which, by exenteration of the orbit, permits access to the entire orbital region of the frontal lobe without the necessity of retracting the brain. Under these circumstances, using either ether or nembutal for light anesthesia, the inhibitory respiratory region is found to be a very small area situated in the gyrus compositus anterior just lateral to the posterior extremity of the gyrus proreus. It is definitely lateral to the sulcus rhinalis and sulcus supraorbitalis.

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[1] Spencer, W. G., *Philos. Trans.*, 1894, B **185**, 609.

[2] Bucy, P. C., and Case, T. J., *J. Nerv. and Ment. Dis.*, 1936, **84**, 156.

[3] Smith, W. K., *J. Neurophysiol.*, 1938, **1**, 55.

[4] Bailey, P., and Sweet, W. H., *J. Neurophysiol.*, 1940, **3**, 276.

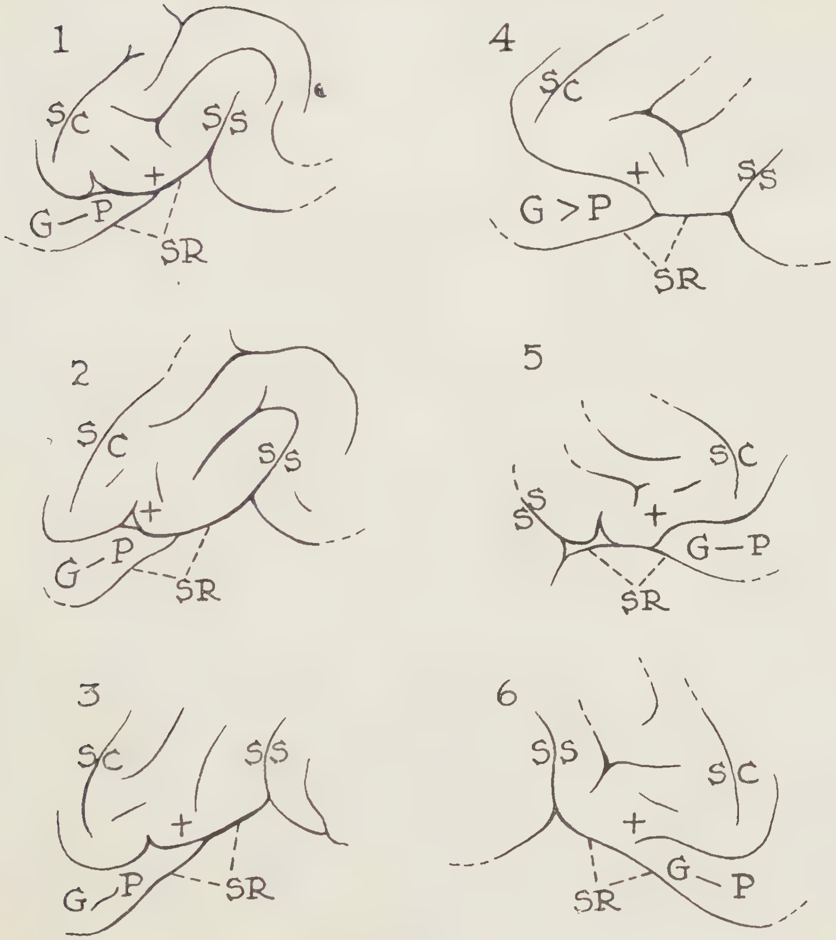

FIG. 1.
The inhibitory respiratory center is marked by a +. *SC*—sulcus coronalis; *SS*—sulcus Sylvii; *SR*—sulcus rhinalis; *GP*—gyrus proreus.

We have never obtained slowing or arrest of respiration by stimulation medial to the sulcus rhinalis. There is considerable variation in the pattern of gyri and sulci in this region of the dog's cortex. The accompanying diagram (Fig. 1) shows the location of the inhibitory respiratory center in 6 dogs. It lies always in the gyrus compositus anterior just posterior to the area indicated by Smith. This seems to be the area 4 c of Klempin.[5] We have never obtained inhibition from any other area of the lateral, orbital or medial surface of the frontal lobe.

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[5] Klempin, D., *J. Psychol. u. Neurol.*, 1921, **26**, 229.

11799

Effect of Thyroidectomy and of Experimental Hyperthyroidism Upon Histamine Content of Rat Tissues.

F. R. Gotzl and C. A. Dragstedt.

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Abelin[1] has recently studied the effects of experimental hyperthyroidism upon the chemistry of the skin in rats, with especial reference to cholesterol, chloride, water and fat. So far as we know, no one has determined the effect of experimental hyperthyroidism upon the histamine content of the skin. Such a determination appeared to be of interest for two reasons. It might add circumstantial evidence to the theory that the histamine content of tissue is the result of local metabolic processes rather than a mere storage of histamine that has been absorbed from the intestinal tract. Also it might reveal a possible explanation for the apparent increased incidence of urticaria and dermographism reported in patients with hyperthyroidism.

In order to check the possibility that changes in the skin histamine depended upon a shifting of histamine from skin to viscera or the reverse, the liver and lungs were examined also. In addition it seemed desirable to contrast the effects of an experimental hyperthyroidism with those of an experimental hypothyroidism.

The histamine content of the skin, liver and lung was determined by Best's[2] method in 3 series of rats, a control group, a group that had been thyroidectomized from 16 to 20 days previously, and a group that had received thyroid daily for 8 to 17 days. The thyroid was administered subcutaneously as a matter of convenience and was given in 10 mg doses.

The results are shown in the accompanying table. The observations indicate that thyroidectomy results in a decrease in the histamine content of the skin and conversely that an experimental hyperthyroidism results in an increased content of histamine. There is some indication that similar changes occur in the histamine content of the liver and lung but in these organs the range of values for normal animals is so wide that it would probably take a much larger number of animals to definitely establish this conclusion. It seems fair to conclude, however, that neither the decrease in skin histamine resulting from thyroidectomy nor the increase in skin histamine occurring after thyroid administration is due to a shift of histamine from skin to other organs and vice versa. The effect of thyroidectomy and of thyroid administration upon the histamine content of the tissues of other laboratory animals is under investigation.

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[1] Abelin, I., *Biochem. J.*, 1940, **34**, 15.

[2] Best, C. H., *J. Physiol.*, 1929, **64**, 256.

TABLE I.
Histamine Content of Rat Tissues.

	Tissue	No. of animals	Histamine base in μg per g of tissue Range	Histamine base in μg per g of tissue Mean
Normal	Skin	10	10.9-16.5	11.5
	Liver	8	>0.2- 2.8	0.8
	Lung	11	3.6-10.9	5.8
Thyroidectomized	Skin	8	2.8- 8.2	4.8
	Liver	9	>0.2- 1.0	0.18
	Lung	9	>0.2-10.7	2.8
Hyperthyroid	Skin	12	10.9-32.9	21.2
	Liver	12	>0.2- 3.0	1.1
	Lung	12	>0.2-12.0	6.4

11800 P

Experimental Analysis of Kuo Vaseline Technique for Studying Behavior Development in Chick Embryos.†

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The purpose of this study is to examine the experimental method used by Kuo[1-5] to study development of embryonic behavior in the chick. The conditions under which Kuo's experiments were performed,[1, 2] were as follows.

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† Complete data will appear in the final article to be published in *The Journal of Genetic Psychology* in the near future.

* Aided by a grant to Doctor Windle from the John and Mary R. Markle Foundation.

[1] Kuo, Z. Y., *J. Exp. Zool.*, 1932, **61**, 395.

[2] Kuo, Z. Y., *J. Exp. Zool.*, 1932, **62**, 453.

[3] Kuo, Z. Y., *Psych. Rev.*, 1932, **39**, 499.

[4] Kuo, Z. Y., *J. Comp. Neurol.*, 1939, **70**, 437.

[5] Kuo, Z. Y., *Psych. Rev.*, 1939, **46**, 93

The shell of the incubating chick's egg was opened in the region of the respiratory air space, exposing part of the inner shell membrane. This was coated with a thin layer of liquid vaseline to render it transparent. The eggs were then observed daily while they incubated upright on their small ends. The impression is given that the same chick could be observed daily from the 2nd, or earliest day of operation, to the 21st, or day of hatching. Except in one paper[6] in which he reported placing only a spot of vaseline on the membrane above the embryo, Kuo has consistently referred to this original procedure.[1, 2] Behavior observed under such conditions he has considered normal.

Because we questioned the physiologic soundness of this procedure, limiting as it does the normal respiratory exchange of the chick, experiments were undertaken to determine the chances of viability and normal development in chicks incubating under these and similar conditions. Kuo did not record the number of chicks which hatched or the number which failed to do so.

Three experiments were run. In the first, 72 eggs were incubated and operated exactly as described by Kuo.[1] The inner shell membranes of random groups of these eggs were coated with a thin layer of melted vaseline on the 3rd, 4th, 7th, 8th, or 11th day of incubation. None of these chicks survived more than 6 days; 72% died within 3 days of operation. Cyanosis was observed in every case. Eight normal controls, unopened and lying on their sides, hatched satisfactorily and developed into normal healthy chicks.

In the second experiment, 44 eggs were prepared in a similar manner, but vaseline was applied only to a small localized area of the inner shell membrane above the embryo on the 3rd or 4th day of incubation. None survived more than 2 days after operation. Three normally incubated unopened controls hatched; one other died of unknown cause on the 11th day.

In the third experiment, the shell at the large end of 37 eggs was painted with clear white shellac on the 1st, 3rd, 4th, 7th or 11th day of incubation. Twenty-six embryos failed to hatch; 87% of these died before the 13th day of incubation. Of the 11 which hatched, only 4 were normal healthy birds. The other 7 were abnormally developed (stunted, club feet, twisted necks, weak legs, etc.) and died within 3 days after hatching. Four unshellacked eggs incubating with the experimental eggs hatched into normal healthy chicks.

The results of the third experiment agree with the early reports of Baudrimont and St. Ange[7] and Dareste[8–10] who found that obliteration of the respiratory air space by varnishing the large end of the shell led to asphyxial death of the embryo. Varnishing over the small end did not affect development in any way.

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[6] Kuo, Z. Y., and Carmichael, L., *J. Psychol.*, 1937, **4**, 343.

The results of oiling the shell membrane are not in agreement with Kuo's reports that chicks develop normally when the inner shell membrane is vaselined. It appears that such a procedure likewise engenders anoxemia. This fact, considered in the light of recent studies on behavioral development in bird and mammalian embryos,[11–12] may explain why Kuo frequenty encountered prehatching respiratory activity and somatic "mass motility" in his specimens.[2–5] Even in the normal, undisturbed, incubating egg an adequate gaseous exchange between the chick and the outside atmosphere is maintained with difficulty.[13] Vaselining the inner shell membrane in the region of the air space, designated by some as the "avian placenta," leads to abnormal, asphyxial behavior patterns.

11801 P

Pathogenicity of "Carrier" Strains of *Endamoeba histolytica* in the Experimental Dog.

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For many years it has been recognized that individuals may harbor *Endamoeba histolytica* without exhibiting clinical symptoms. Such individuals have often been termed healthy carriers. It has been suggested that this organism may not always invade tissue but that it may live in the large bowel as a commensal. Brumpt[1] holds this belief and consequently divides the organism into 2 separate species according to its pathogenicity or non-pathogenicity for experimental kittens. Craig[2] is one of the strongest advocates of the belief that all strains are actually or at least potentially pathogenic to man. He has repeatedly stated that *E. histolytica* is essentially a tissue parasite and that it cannot live and propagate for any length of time in the human intestine without producing some tissue damage.

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[7] Baudrimont, A., and St. Ange, M., *Ann. Chim.* (Phys.), 1847, Series 3, **21**, 195.

[8] Dareste, M. C., *C. R. Acad. Sci.*, Paris, 1855, **41**, 963.

[9] Dareste, M. C., *Ann. Sci. Nat.*, 1855, Series 3 (Zool.), **4**, 119.

[10] Dareste, M. C., *C. R. Soc. Biol.*, 1857, **9**, 99.

[11] Windle, W. F., and Barcroft, J., *Am. J. Physiol.*, 1938, **121**, 684.

[12] Windle, W. F., and Becker, R. F., *Arch. Neurol. and Psychiat.*, 1940, **43**, 90.

[13] Romijn, C., and Roos, J., *J. Physiol.*, 1938, **94**, 365.

* Research Fellow in Tropical Medicine, Tulane University.

[1] Brumpt, E., *Trans. Roy. Soc., Trop. Med. and Hyg.*, 1928, **22**, 101.

The present investigation was designed to determine experimentally the pathogenicity of human "carrier" strains of *Endamoeba histolytica*. By inoculating dogs with cysts or trophozoites of *E. histolytica* obtained from the feces of human "carriers," it was expected that evidence could be obtained with reference to the percentage of animals which became infected, the incubation period, the clinical manifestations of the infection and the amount of pathology produced by the amebae.

The amebic strains selected for experimentation were obtained from a group of orphan children who had never shown symptoms of diarrhea or dysentery. They were thus placed in the so-called "healthy carrier" group of individuals harboring *E. histolytica*. Full-grown dogs were utilized as experimental animals, since the course of the disease in them closely simulates that in man. The dogs were either inoculated intragastrically with cysts or intracecally with trophozoites, which had been isolated in culture.

Out of 26 dogs inoculated with 16 different "carrier" strains of *E. histolytica* all became infected with the organism and all showed amebic lesions at autopsy. This 100% infection is remarkably high when one remembers that these strains are considered by many workers to be non-pathogenic. Walker and Sellards[3] found in their human experiments that the incubation periods varied from 1 to 33 days with an average of 11 days. In the present series of dog experiments the incubation periods varied from 2 to 21 days, with an average of 6 days.

On the basis of objective symptoms, presence of amebae and extent and numbers of lesions, the animals were divided into 4 groups: (1) Acute dysentery, with numerous amebae and extensive lesions (4 dogs); (2) mild, evanescent dysentery, with amebae and lesions (6 dogs); (3) no dysentery, with amebae and lesions (3 dogs), and (4) no dysentery, with no detectable amebae but with lesions (13 dogs).

Three types of gross lesions were observed in the 26 experimentally infected dogs: (1) Extensive superficial denudation of the mucosa; (2) ragged, round and slit-like ulcers; and (3) typical pin-point lesions.

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[2] Craig, C. F., *Amebiasis and Amebic Dysentery,* 1934.

[3] Walker, E. L., and Sellards, A. W., *Phil. J. Sci.,* 1913, **8**, 253.

From the above results it is observed that all of the 16 different "carrier" strains of *E. histolytica* were pathogenic to dogs. Every animal became infected and showed amebic lesions at autopsy. The severity of the infections was different for the various strains and for the individual experimental animal but all strains were shown to be pathogenic and in many cases very active tissue invaders. The gross lesions were very typical of amebic infections but in histological sections amebae were never observed beneath the muscularis mucosae. The average incubation period of 6 days is considerably shorter than the 11 days of Walker and Sellards[3] for their human experiments but this is probably due to the fact that the infections could be detected by cecal aspiration at a much earlier date. If the conditions of an amebic infection in the dog are at all comparable to those in man, it is probable that there is no such thing as a healthy carrier condition in man and that all individuals harboring the organism have amebic lesions in the large bowel.

Conclusions. From the evidence cited above it is highly probable that all "carrier" strains of *E. histolytica* are pathogenic and that "healthy human carriers" as well as those individuals exhibiting clinical manifestations of the disease should be regarded as clinical cases.

11802

Conversion of Dibenzyl Disulfide to Hippuric Acid in the Rat.

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It has been demonstrated that benzyl chloride, S-benzyl-*l*-cysteine and S-benzylglutathione, when administered to animals, yield in the urine N-acetyl-S-benzyl-*l*-cysteine.[1-3] S-benzyl-*d*-cysteine undergoes optical inversion in the rat, via the oxidative deamination, to yield N-acetyl-S-benzyl-*l*-cysteine.[4] The optical inversion of S-

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[1] Stekol, J. A., *J. Biol. Chem.*, 1938, **124**, 129.

[2] Stekol, J. A., *J. Biol. Chem.*, 1939, **128**, 199.

[3] Stekol, J. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 108.

[4] du Vigneaud, V., Wood, J. L., and Irish, O. J., *J. Biol. Chem.*, 1939, **129**, 171.

benzyl-*d*-cysteine to the corresponding acetylated *l* enantiomorph has been shown to occur in man as well as the rat.[5] It was observed, however, that the yields of the benzylmercapturic acid in the urine of rats and man after the ingestion of S-benzyl-*l*-cysteine were much greater than those after the ingestion of S-benzyl-*d*-cysteine.[4, 5] This was attributed to partial destruction of the *d* isomer of S-benzylcysteine at some stage of its metabolism to which, apparently, the corresponding *l* isomer is either immune or exposed to a smaller degree.[5] This intermediary stage of the metabolism of S-benzyl-*d*-cysteine is most probably the formation of S-benzyl-thiopyruvic acid, which was undoubtedly formed *in vivo* before the asymmetric synthesis of N-acetyl-S-benzyl-*l*-cysteine occurred. S-benzyl-thiopyruvic acid is expected to be unstable (no successful synthesis of it has been accomplished as yet) and one of the probable breakdown products of S-benzyl-thiopyruvic acid could be benzyl-mercaptan or dibenzyl disulfide. It has been shown that S-methyl-γ-thio-α-ketobutyric acid easily decomposes in alkaline medium to yield methyl mercaptan.[6] Upon feeding S-benzyl-*d*-cysteine to man, hippuric acid was isolated from the urine in addition to the benzyl-mercapturic acid.[5] If our deductions concerning the instability of S-benzyl-*d*-cysteine *in vivo* are correct, *i.e.* if dibenzyl disulfide is one of the breakdown products of it *in vivo,* then the administration of dibenzyl disulfide to animals should result in the excretion of hippuric acid. The present work demonstrates this fact.

Six adult albino rats were fed 300 mg of dibenzyl disulfide mixed with a complete synthetic casein diet and the urine was collected under toluene over a period of 48 hours. The urine was acidified with HCl to Congo Red paper and extracted with several portions of ether. The ether extract was evaporated to dryness, the residue was dissolved in hot water, decolorized with Norit, filtered and cooled. The crystals were recrystallized three times from hot water. M.P. 187-188° (corrected) ; N, 7.67 ; hippuric acid : M.P. 187-188° ; N, 7.82. To insure the completeness of the extraction, the ether extracted urine was evaporated *in vacuo* at 38° to a syrup, acidified with HCl and extracted with several portions of ethyl acetate. The extract was evaporated to dryness and the residue was purified as was described above. The substance isolated proved to be hippuric acid, N, 7.77, M.P. 188-189° (corrected). Total amount of hippuric acid isolated from the urine of rats which ingested 300 mg of dibenzyl disulfide was 250 mg, or about 71% of the theoretical amount. No N-acetyl-S-benzyl-*l*-cysteine was present in the urine of rats after the ingestion of dibenzyl disulfide.

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[5] Stekol, J. A., *Proc. Am. Chem. Soc.*, 100th meeting, Detroit, Mich., 1940.

[6] Waelsch, H., and Borek, E., *J. Am. Chem. Soc.*, 1939, **61**, 2252.

The results strongly support the suggestion that benzyl mercaptan or dibenzyl disulfide may be formed from S-benzyl-*d*-cysteine *via* S-benzyl-thiopyruvic acid *in vivo*. The results further indicate the possible source of hippuric acid when S-benzyl-*d*-cysteine is ingested by animals. In this connection it is of interest to mention the recent work of Wood and Fieser.[7] These workers synthesized the cysteine derivatives of 1,2-benzanthracene, 10-methyl, 1,2-benzanthracene, 3,4-benzpyrene and 10-methyl, 3,4-benzpyrene. The preparation of the substances was prompted by the suggestion that these carcinogenic agents may form the corresponding mercapturic acids *in vivo*.[8] Wood and Fieser[7] found that these cysteine derivatives easily break down in neutral aqueous medium to yield the corresponding disulfides, but the authors suggested the possibility "that the mercaptan and the cysteine derivatives of the present series may undergo eventual conversion to the mercapturic acids, possibly even through the intermediary of the disulfides." In the light of the present work it would appear, however, that the mercaptan and the cysteine derivatives of 1,2-benzanthracene and 3,4-benzpyrene, especially those of the 10-methyl derivatives, would yield *in vivo*, not the corresponding mercapturic acids, but the corresponding hydroxyl or carboxyl derivatives, either free or conjugated with sulfuric acid or glycine respectively. The above discussion is in accord with the possibility that, in the course of metabolism of certain carcinogenic agents, conjugation with cysteine may occur at some intermediate stage, even though the corresponding mercapturic acids may not be the end products of such metabolism.

Summary. Dibenzyl disulfide was fed to rats and hippuric acid was isolated from the urine and identified by analysis. The results support the suggestion that benzyl mercaptan or dibenzyl disulfide may be formed *in vivo* from S-benzyl-*d*-cysteine *via* S-benzyl-thiopyruvic acid. Certain phases of the metabolic transformation of sulfhydryl and cysteine derivatives of certain carcinogenic compounds are discussed.

The author wishes to express his thanks to Mr. J. Alicino for the microanalytical work.

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[7] Wood, J. L., and Fieser, L. F., *J. Am. Chem. Soc.*, 1940, **62**, 2674.

[8] White, J., and White, A., *J. Biol. Chem.*, 1939, **131**, 149.

11803

Effects of Colchicine Upon Viscosity of the Arbacia Egg.

Karl M. Wilbur. (Introduced by L. V. Heilbrunn)

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The work of Dustin[1] and Lits[2] on the effects of colchicine upon mammalian tissues has stimulated a large number of investigations of the cytological and cyto-genetic effects of this drug on plant and animal material. Nearly all of these studies have demonstrated that colchicine disturbs the normal processes of cell division. In contrast to the many morphological studies relatively little attention has been given to the basic physiological and protoplasmic changes produced by colchicine, changes which must be associated with the characteristic morphological alterations of the mitotic cycle. It was with the purpose of understanding further the action of colchicine on protoplasm that a study of the effects of this alkaloid on the protoplasmic viscosity of the unfertilized and fertilized egg of *Arbacia punctulata* was undertaken.

Methods. Eggs were placed in glass tubes containing 0.73 M sucrose to prevent the eggs from being thrown against the end of the tube and centrifuged in an Emerson hand centrifuge at approximately 2050 times gravity. The viscosity is expressed as the number of seconds of centrifuging necessary to stratify the eggs so that at least 80% but less than 100% showed a hyaline zone whose thickness was approximately one-sixth the diameter of the egg as measured from the periphery.[3] The centrifuging required to attain the end-point in the fertilized colchicine-treated eggs failed to throw down a considerable portion of the cortical pigment, although much of this could be moved by longer centrifuging.

The temperature varied from 21.0 to 25.3 degrees during the course of experimentation, but the variation during any single experiment was less than 2.0 degrees. Since all the experiments were not carried out at the same temperature, at any arbitrary time following fertilization different batches of eggs were in slightly different stages of development. Only batches of eggs which showed more than 95% fertilization were used.

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[1] Dustin, A. P., *Bull. Acad. Roy. de Med. de Belg.*, 1934, **14**, 487.

[2] Lits, F., *Comp. Rend. de la Soc. de Biol.*, 1934, **115**, 1421.

[3] Angerer, C. A., *J. Cell. and Comp. Physiol.*, 1937, **10**, 183.

Colchicine in a concentration of 10^{-3} (*i.e.*, 1.0 g to 1000 cc of sea water) was used in experiments with unfertilized eggs and 0.5 x 10^{-3} for experiments with fertilized eggs. Solutions were made up fresh daily. A concentration of 0.5 x 10^{-3} is sufficiently strong to prevent mitosis from progressing further than the metaphase stage.[4] Fertilized eggs placed in this solution for as short a time as 15 minutes fail to cleave when returned to sea water. In agreement with Nebel it was found that colchicine in a concentration of 10^{-5} will not completely inhibit the cleavage of eggs placed in this solution 5 minutes after fertilization.

Results. Unfertilized eggs. The protoplasmic viscosity of unfertilized eggs immersed in 10^{-3} colchicine is not significantly different from that of eggs in sea water. The viscosity was tested over a period of 3 hours or longer on each of 7 samples of eggs. That the eggs had been affected, however, was demonstrated by immersing eggs for one hour in 10^{-3} colchicine, washing a single drop of these eggs for an hour in 2 changes of sea water of approximately 250 cc each and then fertilizing them. Fertilization membranes were formed, but in 7 experiments not a single egg was seen to have cleaved 4 hours after fertilization. Many of these eggs showed 2 light areas, which may represent daughter nuclei. (Compare Beams and Evans.[5])

Fertilized eggs. Eggs were fertilized in sea water and transferred to 0.5 x 10^{-3} colchicine 5 minutes later. The viscosity was then studied for 2 hours following fertilization.* If one determines the viscosity of normal eggs at various times before first cleavage, the viscosity will be found to increase, then decrease and be relatively low at the amphiaster stage, and finally to increase again before cleavage. (For references see Heilbrunn[6]) And in Table I the viscosity in sea water can be seen to be high 15 minutes after fertilization and to be somewhat lower 30 minutes after fertilization. However, the colchicine-treated eggs do not follow these characteristic viscosity changes. The large increase which normally follows fertilization does not take place in colchicine; and 15 minutes after fertilization the viscosity of the fertilized egg is about the same as

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[4] Nebel, B. R., *Biol. Bull.*, 1937, **73**, 351.

[5] Beams, H. W., and Evans, T. C., *Biol. Bull.*, 1940, **79**, 188.

* To obviate aging of the eggs determinations for all of the intervals given in Table I were never carried out on any one sample of eggs since this would require many hours.

[6] Heilbrunn, L. V., *The Colloid Chemistry of Protoplasm*, Berlin, 1928 Chap. XV.

TABLE I.

Relative Viscosity of Eggs treated with 0.5×10^{-3} Colchicine.

Exp. No.	Unfert. eggs	15 min after fert.		23 min after fert.		30 min after fert.		60 min after fert.	120 min after fert.
		Sea water	Colch.	Sea water	Colch.	Sea water	Colch.	Colch.	Colch.
1.	60	200	70			180	80		
2.	80	190	70			160	110		
3.	60	160	80			120	100		
4.	70	180	60			140	60		
5.	60	200	70			120	100		
6.	40	200	60			160	80		
7.	60-70	180	60			120	100		
8.	60			120	60			80	120
9.	80			180	80			90	140
10.	60			200	70			70	100
11.	50			120	90			120	120
12.	60			140	60			80	70
13.	50			160	90			80	90
14.	40			180	60			100	120

that of the untreated unfertilized egg. The viscosity was found to be somewhat higher 30 minutes after fertilization in 6 of 7 cases, but a high value is not reached even after 2 hours. Beams and Evans[5] found that *Arbacia* eggs placed in 0.0002 M colchicine 10 minutes after fertilization for a period of 10 minutes are stratified more easily than eggs which have developed for 10 minutes in sea water. This result also indicates a lower viscosity for the eggs in colchicine.

The appearance of the "streak" so characteristic as a stage before the amphiaster—and one undoubtedly associated with spindle formation—is absent in colchicine-treated eggs. This is in agreement with the well-known effect of colchicine in inhibiting the formation of the achromatic apparatus.

The fusion of the pronuclei and the breakdown of the zygote nucleus are slowed in eggs whose viscosity has been kept low by colchicine. Eggs were fertilized and 5 minutes later some of the eggs were transferred to 0.5×10^{-3} colchicine. When the eggs in sea water were seen to have reached the amphiaster stage both the eggs in sea water and those in colchicine were centrifuged at approximately 2050 times gravity for 5 minutes. Most of the colchicine-treated eggs showed one or two light areas at the centripetal pole, indicating that the pronuclei had not fused (eggs with two light areas) or that they had fused but that the nuclear membrane had not as yet broken down (eggs with a single light area). Apparently colchicine has not completely inhibited the eggs in these stages, for if the eggs are centrifuged after 2 hours or longer following fertilization, the light areas are not seen, indicating that fusion of pronuclei and nuclear breakdown have occurred.

Dustin and his collaborators[7,8] believe that colchicine initiates mitosis as well as arresting the process once it has begun. Others do not agree that the number of cells in mitosis is increased by the action of colchicine.[9–12] The eggs of *Nereis limbata* were examined for mitotic activity as indicated by germinal vesicle breakdown after 3¾ to 5 hours in the following concentrations of colchicine: 10^{-4},

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[7] Dustin, A. P., Havas, L., and Lits, F., *Comp. Rend. Assn. des Anat.*, 1937, **32**, 177.

[8] Dustin, A. P., *Comp. Rend. Assn. des Anat.*, 1938, **33**, 205.

[9] Ludford, R. J., *Arch. f. exp. Zellforsch.*, 1936, **18**, 411.

[10] Brues, A. M., *J. Physiol.*, 1936, **86**, 63P.

[11] Mangenot, G., *Comp. Rend. de la Soc. de Biol.*, 1938, **128**, 501.

[12] Gavaudan, P., and Gavaudan, N., *Comp. Rend. de la Soc. de Biol.*, 1938, **128**, 714.

10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10}. Seven series of experiments in which each of these concentrations was employed were carried out, and in no case was there any germinal vesicle breakdown.

Discussion. Apparently certain processes which are initiated following sperm entry and which result in increased viscosity are inhibited by colchicine. In the unfertilized egg these processes may be absent, with the result that there is no alteration of viscosity by colchicine. That is, colchicine seems to have no primary viscosity effect on protoplasm, as shown by its failure to change the viscosity of the unfertilized egg appreciably, but acts by inhibiting processes which do produce viscosity changes. Quite probably the failure of the spindle to form in the presence of colchicine is in some way related to this inhibition. The dependence of spindle formation on an increase in viscosity was expressed by Heilbrunn,[13] who found that substances which prevented an increase following fertilization also prevented formation of the spindle. Beams and Evans[5] have suggested the same for colchicine. The biochemical effects of colchicine are too incompletely known to permit any statement of the probable nature of the inhibition, but perhaps enzymatic changes are involved. The increase in viscosity which takes place prior to cleavage might also be inhibited.

Mention has been made of the fact that in colchicine division processes proceed to the point of nuclear breakdown. Now normal fertilized eggs which have reached this stage have passed through a period of high viscosity. If it is true that colchicine-treated eggs with their low viscosity have passed through a stage which in the normal has a high viscosity, then, of course, the characteristic increase in viscosity of the normal egg cannot be considered indispensable to certain early phases of mitosis.

Summary. Colchicine in a concentration of 10^{-3} in sea water does not appreciably alter the viscosity of the unfertilized *Arbacia* egg. The increase in viscosity which normally follows fertilization is inhibited by colchicine in a concentration of 0.5×10^{-3}. Fusion of the pronuclei and nuclear breakdown are delayed in colchicine-treated eggs. Colchicine in concentrations of 10^{-4} to 10^{-10} does not initiate mitosis in the *Nereis* egg.

I wish to acknowledge the helpful suggestions of Dr. L. V. Heilbrunn and Dr. C. A. Angerer.

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[13] Heilbrunn, L. V., *Anat. Rec.*, 1917, **11**, 487.

11804

Incidence of *Trichinella spiralis* in Garbage-Fed Hogs in San Francisco.*

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Hall and Collins[1], summarizing published incidences of *Trichinella spiralis* in human autopsies in the United States, reported a total of 1,778 examinations with 12.5% positive. (More recent reports[2] place this figure at about 18%.) They noted that Queen[3] had found an incidence of 27.6% in Boston and McNaught and Anderson[4] 24% in San Francisco while Hinman's[5] survey of New Orleans had shown only 3.5% with other cities taking an intermediate position. They correlated the incidence of trichinella in man and that in the hogs from which they obtained their pork. Hall[6] and Schwartz[7] reported incidences of 1 to 1.5% of live trichinella in grain-fed hogs and 4.8 to 5% in garbage-fed hogs. Hogs in the vicinity of Boston fed offal from slaughtered hogs had previously shown as high as 18% infected. Hall and Collins suspected that the garbage feeding establishments supplying pork to Boston were largely responsible for the relatively high incidence of trichinosis in that area with a similar situation in San Francisco. In the South most hogs run at large in the fields and woods until ready for fattening on peanuts previous to slaughter and are not exposed to infection with trichinella. The incidence of infection in these hogs has been shown to be from .1 to .8% and probably accounts for the low figures for man in New Orleans and the more recent 2.8% for North Carolina.[8]

Having determined the incidence of trichinella infestation in human diaphragms in San Francisco[4] we were desirous of obtaining similar information regarding the hogs sold for consumption in this area. In May 1938 we began examining garbage-fed hogs received by San Francisco abattoirs. Diaphragms from hogs from each hog ranch were submitted for examination.

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* This survey was undertaken at the request of the Department of Public Health of San Francisco and the expenses were defrayed by the South San Francisco Packing and Provision Company and the H. Moffat Company of this city.

[1] Hall, M. C., and Collins, B. J., *Pub. Health Rep.*, 1937, **52**, 468.

[2] Pollock, R. C., *J. A. M. A.*, 1940, **114**, 35.

[3] Queen, F. B., *J. Parasitol.*, 1931, **18**, 128.

[4] McNaught, J. B., and Anderson, E. V., *J. A. M. A.*, 1936, **107**, 1446.

[5] Hinman, E. H., *New Orleans M. and S. J.*, 1936, **88**, 445.

[6] Hall, M. C., *Ann. Rep. Chief of B. of Animal Ind.*, 1935, p. 51.

[7] Schwartz, B., *Idem*, 1936, p. 56.

[8] Harrell, G. T., and Johnston, C., *So. Med. J.*, 1939, **32**, 1091.

Methods. The diaphragms were weighed, ground in a meat chopper, and subjected to 2 well recognized procedures for examining meat for trichinella. A quarter of a gram of each was pressed thin between glass slides and examined under the microscope using a magnification of 20. The remainder of each diaphragm was digested by artificial gastric juice and the concentrated sediment searched for the parasites.[4]

Results. Diaphragms from 495 hogs were received in 52 lots. There were usually 10 diaphragms per lot. All specimens in each lot were from the same hog ranch. The weight of the individual specimens varied between 5 and 100 g, averaging 21 g. Fully 85% weighed between 10 and 35 g. Twenty of the 495 diaphragms contained *Trichinella spiralis* showing an incidence of 4.04%.

The 20 positive specimens were from 13 lots. Eight contained one positive diaphragm each, three contained 2 each, and 2 contained 3 each. Although the examination of 10 diaphragms does not yield figures which are statistically valid it is noteworthy that the positive incidences of 8 ranches were 10%, 3 were 20%, and 2 were 30%. Table I details the findings for the positive diaphragms. Fifty-five per cent of the positive specimens contained less than one parasite

TABLE I.
Findings for Positive Cases of *Trichinella spiralis.*

Positive No.	Meat Wt, g	Findings by: Press	Findings by: Digestion	Total No. of Parasites	No. per g of meat
1	20	—	+	18	.9
2	12	—	+	402	33.5
3	22	+	+	657	29.8
4	25	—	+	8	.3
5	100	—	+	480	4.8
6	31	+	+	20,230	652.5
7	20	+	+	752	37.6
8	20	—	+	18	.9
9	13	—	+	6	.5
10	12	+	—	2	.2
11	26	+	+	530	20.4
12	28	—	+	14	.5
13	26	—	+	11	.4
14	16	+	+	54	3.3
15	20	—	+	13	.6
16	12	—	+	10	.8
17	16	+	+	102	6.4
18	14	—	+	11	.8
19	32	+	+	1,657	51.8
20	20	—	+	1	.05

per gram of muscle, 15% contained less than 10, 25% contained less than 100 and 5% contained less than 1000 per gram. No. 10 showed only 2 calcified cysts in the pressed specimen. Since no larvæ were found in the sediment it is assumed that whatever cysts were present in the remainder of the meat were completely digested and no live larvæ remained. All other specimens yielded living larvæ.

Only 8 of the 20 positive diaphragms showed trichinella by the press method while 19 were positive by digestion. The press procedure is adequate in detecting moderately heavy infections but not the light ones. It is valuable in detecting the calcified encysted parasites which are eventually digested and do not appear in the concentrated sediment. Naturally if larger quantities of muscle had been used in the press preparations a larger percentage of positives would have been found.

Discussion. The 4.04% incidence of trichinella which we found in 495 garbage-fed hogs raised in the San Francisco area closely approximates the percentages obtained by Hall and Schwartz for garbage-fed hogs in other parts of the United States. It is considerably lower than the 15% found by Hobmaier and Geiger[9] in 93 garbage-fed hogs in this area. The lower incidence in our survey completed in 1940 may have been in part due to the introduction of rodent control at the hog ranches through the efforts of the San Francisco Department of Public Health following the survey of Hobmaier and Geiger.

We find that the incidence of trichinosis in hogs is considerably less than that in humans (24%) in this area. In regions like Boston and San Francisco where the county hospital material comprises a high percent of immigrants it can not be assumed that all infections were acquired locally. Although only one out of 25 hogs contained the parasite, the meat from a single infected hog could infect many people. It is usual in large meat packing establishments to mix trimmings from many hogs in the preparation of sausage or other pork products, so that the meat from one badly infected hog could contaminate large quantities of the final product. If this product was consumed raw or improperly prepared many people could become infected. The dilution of the infected meat would however diminish the opportunities for severe or fatal infections of man.

It is apparent that an effective method of preventing trichinosis in man is to eliminate the infection in hogs. Alicata[10] in 1938 found only one trichinous animal out of 299 garbage-fed hogs in the

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[9] Hobmaier, M. D., and Geiger, J. C., *Am. J. of P. H.*, 1938, **28**, 1203.

[10] Alicata, J. E., *Public Health Rep.*, 1938, **53**, 384.

Hawaiian Islands, an incidence of .3%. These hogs were kept in concrete or wooden pens and fed cooked garbage. In this same area 13.6% of 44 wild hogs were infected with trichinella. Cameron[11] found only .2% of 955 diaphragms from garbage-fed hogs in Canada contained this parasite. In Canada all garbage-feeding hog ranches are required to be licensed and inspected and all garbage is cooked prior to feeding. The low figures found in the Hawaiian Islands and Canada suggest strongly that the cooking of garbage and careful inspection of hog ranches are sound procedures for preventing much of the trichinosis in hogs.

Summary. The diaphragms of 495 garbage-fed hogs received in 2 abattoirs of San Francisco showed 20 or 4.04% infected with *Trichinella spiralis.* This figure is comparable with similar surveys of garbage-fed hogs in other areas of the United States. It is 20 times higher than found in garbage-fed hogs in Canada where garbage is cooked before feeding.

11805

Recovery of Virus Morphologically Identical with Psittacosis from Thiamin-Deficient Pigeons.

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The virus to be described here was recovered from young pigeons purchased in South Carolina. These pigeons, which were between 6 and 8 weeks old when received, were kept on a diet of mixed grain for one or 2 weeks, and then put on the following diet: Vitamin-free casein—180 g; cod liver oil—40 g; peanut oil—50 g; salt mixture—40 g and corn starch—580 g. Eighteen to 20 g of the above mixture was introduced daily by tube into the crops of the birds. The original purpose of these experiments was to study the changes produced in the central nervous system of the birds on this diet. These results have already been reported.[1]

In the majority of birds kept on the above diet, vomiting occurred on and after about the eighth day, opisthotonus began usually on the fourteenth day, and death rarely occurred before the twenty-first day. Occasional birds, however, developed leg weakness or opisthotonus between the sixth and tenth days, and died a day or two thereafter. In such birds, fibrinous peritonitis and pericarditis, with or without enlarged hemorrhagic livers, were occasionally observed. Since these organs and exudates appeared sterile when planted in ordinary bacteriological media, mice were injected intracanially in an attempt to determine the cause of the condition.

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[11] Cameron, T. W. M., *Canad. J. Res.*, Sect. D, 1940, **18**, 83.

[1] Swank, R. L., *J. Exp. Med.*, 1940, **71**, 683.

From the exudate or ground-up organs of 4 pigeons a serially transmissible virus was recovered by this method which killed mice 2 to 4 days after intracranial injection. Histological examination of the brains of these mice showed a well marked chorio-meningo-encephalitis, mononuclear cells predominating in the inflammatory exudate. Within the mononuclear cells, large numbers of minute intracellular bodies having the typical appearance and arrangement of the bodies seen in psittacosis were observed. Similar bodies were later found in large numbers in the exudate on the surface of the livers in 2 of the pigeons from which the mice were injected.

Nature of Virus. Morphologically the picture was identical with that of psittacosis. The appearance of the intracellular bodies in this disease is so well known that it seems unnecessary to illustrate it in this paper. In view of the fact that the appearance of cells infected with this virus is unique and not duplicated in any other infection, it seemed justifiable to conclude that our virus was closely related to, if not identical, with that of psittacosis. Cross-immunity tests with known psittacosis virus were, however, not carried out because of the fact that the virus was lost through too great reliance on its preservation in glycerine. (In early experiments it persisted, with some loss of virulence, for more than 8 weeks.)

Biologically our virus differed from typical strains of psittacosis virus in the fact that it was pathogenic for mice only when introduced intracranially. Intraperitoneal or subcutaneous injection of large doses of the virus had no apparent effect. Whether this atypical behaviour was due to a modification of the virus or merely to a partial loss of virulence was unfortunately not determined. Recent attempts to obtain the virus again from pigeons purchased from the same dealer have not been successful.

Frequency of the Infection. From a total of 400 pigeons on the deficient diet, the presence of the infection was proven by mouse injection in only 4 instances. In 16 other birds, however, presumptive evidence of infection, in the form of hemorrhagic livers or fibrinous peritonitis, was noted. Exact figures cannot be given because all birds were not examined post-mortem, but less than 10%, and probably no more than 5% of the deficient pigeons showed apparent infection. No tests for inapparent infection were made. Six of the presumably infected birds and 2 of those proven infected, were found in one group of 24 pigeons purchased in March, 1938.

Relation of the virus infection to the dietary deficiency. All birds studied were in apparently good health when received, and remained in good health during the preliminary period of one to 2 weeks on normal diet. Moreover, no birds died during their first 4 or 5 days on the diet, but all deaths from the infection occurred before the twelfth day on the diet. The relative constancy of the period of deficient diet preceding manifestations of infection suggested a relationship between the deficiency and the infection similar to that shown to exist between riboflavin deficiency and experimental typhus fever in the rat.[2]

Summary and Conclusions. A virus closely related to, if not identical with that of psittacosis was isolated from about 5% of 400 pigeons on a thiamin-deficient diet. The observations made suggest: 1. That pigeons should be added to the list of birds naturally infected with psittacosis virus and therefore of possible importance in the epidemiology of the disease, and 2. That thiamin deficiency may be capable of changing a latent infection with psittacosis virus into an active stage, with consequent danger of spread to other birds and to man.

Acknowledgment should be made of the assistance of Dr. T. M. Rivers who kindly examined our sections and stated that the intracellular bodies seemed identical with those of psittacosis.

11806 P

Effect of Undernutrition on Thyroid Tissue Respiration of Guinea Pig.*

D. J. STEPHENS AND IRVIN J. BELASCO. (Introduced by W. S. McCann.)

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In a previous publication[1] it has been shown that undernutrition in the guinea pig results in marked structural changes in the thyroid

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[2] Pinkerton, H., and Bessey, O. A., *Science*, 1939, **89**, 368.

* This investigation was aided by grants of the Committee for Scientific Research of the American Medical Association and the Committee on Endocrinology of the National Research Council.

[1] Stephens, D. J., *Endocrinology*, 1940, **26**, 485.

TABLE I.
Oxygen Consumption of Thyroid Glands of Normal and Undernourished Guinea Pigs.

No.	Original wt, g	Final wt, g	% gain or loss body wt	Wet wt 2 thyroids, mg	Dry wt 2 thyroids mg	Dry wt, %	Mm³ O₂ per mg dry tissue, per hr	Mm³ O₂ per hr, entire 2 thyroids	Thyroid O₂ consumption, mm³ per kg body wt per hr
Normal									
E-48	270	305	+13	44.0	5.45	12.4	4.70	25.2	82.6
E-49	295	335	+13	47.8	7.08	14.8	3.76	26.2	79.1
E-50	285	325	+14	43.6	5.14	11.8	3.49	17.9	55.0
E-51	270	305	+13	33.0	4.53	13.7	3.58	16.2	52.6
E-52	270	300	+11	33.9	4.06	12.0	4.94	20.1	67.1
Avg	278	316	+13	40.4	5.25	13.0	4.09	21.2	67.3
Undernourished									
E-42	310	265	—14	31.4	3.69	11.7	3.93	14.5	54.7
E-43	290	230	—21	25.0	3.46	13.9	3.11	10.7	46.5
E-44	290	240	—18	28.1	3.36	12.0	3.26	10.9	45.4
E-45	305	245	—20	31.0	5.28	16.6	2.46	13.0	53.0
E-46	285	230	—20	23.9	3.10	13.0	3.21	9.9	41.2
Avg	296	242	—18	27.9	3.78	13.5	3.19	11.8	48.2
							—22%	—44%	—28%

characterized by atrophy and flattening of the acinar epithelium and retention of colloid, suggesting a resting, inactive gland. This communication is a preliminary report of a study of the thyroid tissue respiration in such animals.

Procedure. Mature, female guinea pigs, weighing approximately 300 g, were selected for study. Details of the care and feeding of these animals have been described.[1] Undernutrition was produced by feeding a quantitatively inadequate diet resulting in a loss of approximately 20% of body weight in a period of 2 weeks. At the end of the experimental period the animals were killed by a blow on the head. A group of animals receiving a full diet was killed while gaining weight to serve as normal controls. The thyroid glands were removed immediately, weighed, and thin slices of tissue removed for the determination of oxygen uptake by the use of the Warburg constant volume manometers, according to the procedures previously described by Belasco and Murlin.[2] Another portion of thyroid of each animal was reserved for histological study; the remainder was then weighed again and dried to constant weight.

Results are summarized in Table I. The undernourished animals had lost an average of 18% of original body weight in a period of 2 weeks. Both the wet and dry weights of the thyroids of the undernourished animals were considerably reduced. The oxygen consumption of thyroid calculated as cu mm per mg of dry tissue per hour showed a significant reduction (—22%) in the undernourished animals. When calculated as the total oxygen consumption of the entire 2 thyroids, a reduction of 44% was found in the undernourished animals. The oxygen consumption of thyroid tissue was also calculated in relation to the total body weight of the animals. The thyroid O_2 uptake in cu mm per kg of body weight per hour was 28% less in the undernourished animals than in the normal controls.

Conclusions. The oxygen consumption of the thyroid glands of undernourished female guinea pigs was significantly reduced.

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[2] Belasco, Irvin J., and Murlin, J. R., *Endocrinology*, in press.

11807 P

Sulfapyridine in Experimental Bacillus Pyocyaneus Infection of the Cornea.*

HAROLD H. JOY. (Introduced by M. S. Dooley.)

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The purpose of this investigation was to determine whether or not the oral administration of sulfapyridine would prevent or influence favorably the infection of rabbit's cornea produced by *Bacillus pyocyaneus.*

The corneæ were inoculated with a 24-hour broth culture of the organism after superficial abrasion. The drug was administered twice daily, at 9:00 A.M. and 5:00 P.M. The dosage for each feeding was 0.5 g/kg the first day, 0.35 g/kg the second day, and 0.25 g/kg thereafter. The soluble sodium sulfapyridine† was used for the sake of greater accuracy in determining dosages. Fifteen rabbits which died or were injured during the course of treatment are not included in this report.

A small benign ulcer appeared within a few hours after inoculation in all rabbits receiving 2 prophylactic doses of sulfapyridine during the preceding 24 hours. In one group of these treatment was continued for from 1½ to 6 days. In 25 eyes the ulcer healed with little or no extension in from 1 to 3 days, but in 2 eyes it continued to progress. In another group treatment was continued for one day or less, at which time the ulcers appeared to be healed. Five of these eyes remained unchanged but in 5 others the ulcer became active after a quiescent period of 24 hours and progressed through a typical course of pyocyaneus infection. Eight eyes were inoculated concurrently with the beginning of treatment which was continued for 2½ days. Mild ulcers developed and healed within 24 hours.

In 2 groups of rabbits treatment was instituted after the appearance of the ulcer and was continued for 6 days. In the first group, in which treatment was begun 6 hours after inoculation, the course was usually mild. In 34 of 46 eyes the maximum inflammation occurred within about 12 hours, and in all but 10 of these the cornea had apparently healed within 24 hours. Altogether, 40 eyes were healed at the end of 6 days. In 6 eyes (13%) the inflammation persisted up to 17 days. A hypopyon appeared in only 5 eyes (11%) and in none did the cornea rupture. In 40 eyes (87%) the resultant scar did not exceed 2 mm in diameter.

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* The expenses of this investigation were defrayed by a grant from the Hendricks Research Fund, Syracuse University College of Medicine. Miss Alice Westcott was technical assistant.

† Donated by Merck & Co., Rahway, N. J.

In the second group of 82 eyes, in which treatment was begun 18 hours after inoculation, the course was usually more severe and the outcome less favorable. The maximum inflammation appeared later and the duration was more prolonged, being over 7 days in 37 eyes (45%). A hypopyon was present in 27 (33%) and the cornea ruptured in 7 (8.5%). The resultant scarring was generally more extensive than in the first group. In 28 eyes (34%) it did not exceed 2 mm in diameter, but in 20 eyes it exceeded 7 mm.

In 82 control eyes the infection occurred in the form of a rapidly progressing superficial ulcer, or less frequently as a ring abscess. A hypopyon, usually large, appeared in 62 eyes (75%) and the cornea ruptured in 46 (56%). The duration of inflammation ranged from 7 days in a few cases to as long as 3 weeks. There were no instances of panophthalmitis. The resultant scar occupied over half the cornea in 72 eyes and in all but 8 of these virtually the entire cornea was involved.

It may be concluded, therefore, that sulfapyridine had a significant effect in the prophylaxis and treatment of experimental pyocyaneus ulcer.

11808 P

Pathological Changes in the Gallbladder Wall Due to Action of Bile.

NATHAN A. WOMACK AND EUGENE M. BRICKER. (Introduced by E. A. Graham.)

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For a long time the local cytotoxic effect of bile and many of its components on tissue has been recognized. With the exception of the more resistant and adapted mucosa of the intestinal tract and the excretory ducts of the biliary tract, there are few instances in which the production of cellular injury does not occur when bile comes in contact with such tissue. This local action is to a large extent dependent upon the concentration of the bile or the several components of bile. For the most part it presents itself as edema, increased capillary permeability with extravasation of blood and direct tissue necrosis with fibroblastic proliferation and round cell infiltration chiefly of the monocytic series. This can be easily demonstrated when bile of various concentrations is injected intramuscularly or subcutaneously and the tissue studied at various time intervals.

Because of the failure of a purely bacteriological explanation of the etiology of cholecystitis to satisfy many of the experimental and clinical aspects of this disease, it has occurred to us that the local effects of bile might be of importance in this condition. That bile may act in this capacity in the production of cholecystitis has previously been suggested by Aronsohn and Andrews[1] and by Ravdin[2] and his co-workers. These investigators injected bile or bile salts into the gallbladder through the cystic duct or common duct and observed acute inflammatory changes in the wall. Cole[3] observed changes in the gallbladder wall following partial cystic duct obstruction. Because of the fact that acute cholecystitis is very rarely seen in the absence of cystic duct obstruction, it occurred to us that this factor must also be considered in the etiological aspects of this disease.

Accordingly, cystic duct obstruction with the injection of bile and several of its components was attempted. The animals were treated by ligation of the cystic duct, ligation of the cystic duct with replacement of the gallbladder bile by an equal amount of physiological salt solution and replacement of gallbladder bile by various concentrations of dog bile, commercial dried bile, cholesterol emulsions, sodium glycocholate and sodium deoxycholate with and without cystic duct obstruction. All injections into the gallbladder were made through the cystic duct in order that there should be no mechanical trauma to the gallbladder wall. In order to eliminate this mechanical trauma further that portion of the gallbladder adjacent to the liver was studied in detail giving an area of tissue far removed from any site of trauma. The animals were sacrificed at various intervals ranging from 24 hours to several months. Fifty dogs and 14 rabbits were used. Because of the closer resemblance of the dog's gallbladder to that of the human it its function and in the type of bile present, this animal was considered best suited for such a study. The results that we wish to report may be summarized as follows.

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[1] Aronsohn, H. G., and Andrews, E., *Surg., Gyn. and Obst.*, 1938, **66**, 748.

[2] Markowitz, J., *Textbook of Experimental Surgery*, William Wood and Co., Baltimore, 1937, p. 264.

[3] Cole, Warren H., personal communication.

(1) With occlusion of the cystic duct after the gallbladder has been emptied and washed of bile and the lumen filled with physiological salt solution no pathological change of importance was noted in the gallbladder wall.

(2) Occlusion of the cystic duct without disturbing the contents of the gallbladder resulted in a moderate amount of edema, round cell infiltration and fibrosis of the gallbladder wall.

(3) Occlusion of the cystic duct with replacement of the gallbladder bile by a solution of commercial dried bile resulted in the various degrees of inflammation that are encountered clinically in cholecystitis depending, however, upon the concentration of the bile solution. Where this concentration was as great as twice that of normal gallbladder bile, complete necrosis of the gallbladder was often encountered. If the cystic duct was not occluded the observed changes were slight and for the most part transient.

(4) When concentrated dog's bile was used a similar change was encountered which likewise seemed to be dependent upon the concentration of the bile. Where this concentration was as great as twice that normally seen, complete necrosis of the gallbladder wall was often encountered. At times where such necrosis was not seen there was often seen either a precipitation in the form of small dark granules of portions of the bile solids or there was an extraordinary out-pouring of mucus with mucus gland hyperplasia on the part of the gallbladder epithelium.

(5) Similar types of reaction have been given by many components of bile. Among those studied have been sodium deoxycholate, sodium glycocholate and cholesterol emulsions with various concentrations as well as cholesterol dissolved in gallbladder bile. These reactions have not all been identical in degree, sodium deoxycholate being the most destructive often resulting in gangrene.

(6) Studies of human gallbladders removed at operation show inflammatory changes identical to the ones that we have produced experimentally in dogs.

From these studies we may conclude that while cholecystitis may be the result of primary bacterial invasion in certain instances it may also occur as a result of cystic duct obstruction, the intensity of the inflammation being dependent upon the composition of the bile imprisoned. This inflammatory process may also be modified by certain protective mechanisms on the part of the gallbladder epithelium and by secondary bacterial invasion.

11809 P

Factors Influencing the Inactivation of the Rabbit Papilloma Virus by X-Rays.

William F. Friedewald and Rubert S. Anderson. (Introduced by Peyton Rous.)

From the Laboratories of the Rockefeller Institute for Medical Research and the Memorial Hospital, New York City.

Lacassagne found several years ago that the rabbit-papilloma virus is notably resistant to X-rays,[1] and Syverton has more recently reported that a dose of 14,000,000 r was required to abolish the infectivity of a cell-free suspension of the papilloma virus[2] though 1,000,000 r or less sufficed to inactivate certain other viruses and bacteria. In experiments undertaken lately for other purposes we have noted that the amount of irradiation required to inactivate the papilloma-virus is influenced by the virus concentration, as also by extraneous material present along with it in the irradiated suspensions. Berkefeld filtrates, which contained the virus in large amount, were rendered completely non-infectious upon exposure to 2 to 4 million r of irradiation, while only 400,000 to 800,000 r was required when the virus had been partially purified by repeated differential centrifugations.

Virus was obtained by grinding infectious cottontail rabbit papilloma-tissue and extracting it in 10 or 20 volumes of isotonic saline and centrifuging at about 4,400 rpm for 20 minutes in an angle centrifuge, with filtration of the supernatant fluid through Berkefeld V candles. Partially purified suspensions of the virus were prepared by centrifuging the filtrate at 30,000 rpm for 1 hour in an air-driven centrifuge, after which the supernatant fluid—which contained no detectable virus but much extraneous protein, as indicated by nitrogen-determinations—was removed. The small pellet of sediment was then resuspended in isotonic saline to the original volume and spun at about 4,400 rpm for 20 minutes. The high-speed centrifugation was then repeated, the pellet of sediment resuspended in saline, and another low-speed centrifugation done to remove gross particles. The final virus suspensions were very faintly opalescent and contained less than 1% as much nitrogen as the whole filtrate.

Twenty to 30 cc of the virus suspension was irradiated in discoid pyrex flasks, 1.8 cm deep with thin flat top and bottom. They were placed half way between 2 X-ray tubes at a distance from each target of 10 cm. To control the temperature the flask was placed in a Petri dish containing water and the air over it was circulated with a fan. The temperature of the water bath never rose above 30°C. The X-rays were not filtered. The tubes were run at 30 milliamperes and at a peak voltage of 180 to 190 kilovolts. The intensity was 6,200 r per minute and the half value layer of radiation was 0.19 mm of copper. The larger doses of irradiation were of necessity intermittent and the virus specimens were kept in the icebox between exposures to the X-rays. Small samples of the virus fluid were removed at intervals during the irradiation and tested for infectiousness by rubbing them into the freshly scarified skin of domestic rabbits. Control portions of the virus solutions submitted to identical conditions, but not irradiated, remained highly infectious.

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[1] Lacassagne, A., *Compt. rend. Soc. biol.*, 1936, **123**, 736.

[2] Syverton, J. T., Third International Cancer Congress, Atlantic City, New Jersey, 1939, 176.

Ten comprehensive experiments have been made, in which 4 virus filtrates were used and 7 partially purified virus suspensions procured from the papillomas of as many different cottontails. The findings have been consistent. Only a very small proportion of the virus in Berkefeld filtrates remained infectious after 1,000,000 r of irradiation, the irradiated material giving rise to less than 1/20th as many papillomas as the controls; and 2 to 4 million r rendered the filtrates completely innocuous. Suspensions of virus partially purified by 2 differential centrifugations, as described above, were almost completely inactivated by 100,000 r and completely so by 500,000 to 800,000 r. Whole filtrates or purified suspensions containing much virus have invariably required more irradiation to abolish the infectivity than comparable preparations containing less virus. This apparent influence of virus concentration is probably due to the use of total inactivation as the quantitative measure of radiation effect.

Normal rabbit serum was added to a purified virus suspension prior to irradiation to learn whether it would have a protecting effect. It was found that the virus suspended in 10% normal rabbit serum retained about half of its infectivity after exposure to 100,000 r and about 10% after 800,000 r, whereas the same virus suspended in saline was rendered almost completely innocuous by 100,000 r and was completely non-infectious after 400,000 r. In 2 further tests the non-infectious supernatant fluids removed from virus filtrates after centrifugation at 30,000 rpm for 1 hour were found to protect purified virus suspended therein instead of in saline, these suspensions requiring about as much irradiation to abolish their infectivity as the original virus filtrates.

It is common knowledge that extraneous proteins protect bacteria and viruses against heat and ultraviolet light, and we have found that the papilloma-virus in crude suspension resists more heat[3] and ultraviolet light[4] than after Berkefeld filtration or partial purification by differential centrifugation. It seems probable that the protection of the papilloma-virus from X-rays by normal serum or by extrinsic material present in papilloma-extracts is also due to extraneous protein. Further work must decide what the findings imply as regards the mode of action of X-rays on biologically active materials.

11810 P

Minimum Daily Requirement of Rabbits for α-Tocopherol.

S. H. EPPSTEIN AND S. MORGULIS. (Introduced by E. S. Guzman Barron.)

From the Department of Biochemistry, College of Medicine, University of Nebraska, Omaha, Nebraska.

During the past year we have been studying the vitamin E requirement of rabbits for the cure and prevention of nutritional muscle dystrophy. Recently, MacKenzie and McCollum[1] reported that the rabbit requires 0.7 to 1.0 mg of α-tocopherol per kg of body weight, but this does not quite agree with our findings. In our experience, rabbits made dystrophic on the Goetsch-Pappenheimer diet 13 were cured by the daily oral administration of synthetic dl-α-tocopherol acetate in quantities ranging from 0.18 to 1.0 mg per kg of body weight, with most of the cures resulting from doses of 0.2 to 0.5 mg (as free alcohol).

We found that a definite correlation exists between the higher requirements and the *total* α-tocopherol intake. When animals with an apparently high vitamin E requirement were subjected to a new test on a smaller *total* tocopherol intake, their requirement dropped to a lower level (values ranging from 0.2 to 0.4 mg per kg). These findings suggest that the higher values, as well as the wider range of variation in the requirement values, are not so much an outcome of individual variability in the need of the tissues for vitamin E as of variations in the efficiency of absorption, in ability for storing and in the rate of destruction of the vitamin.

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[3] Friedewald, W. F., and Kidd, J. G., *J. Exp. Med.*, 1940, **72**, 531.

[4] Unpublished experiments.

[1] MacKenzie, C. G., and McCollum, E. V., *J. Nutrition*, 1940, **19**, 345.

In addition to the experiments with diet 13, we also fed rabbits our diet X 113 from which vitamin E is removed by an exhaustive extraction with lipid solvents. Rabbits on this diet become dystrophic as readily as on diet 13, in which vitamin E is destroyed by oxidation with $FeCl_3$ and rancid fat. We treated rabbits made dystrophic on diet X 113 with much smaller *total* amounts of α-tocopherol than were used with most animals fed diet 13. In the former the total amount administered did not exceed 60 mg per rabbit, while in the latter quantities up to 222 mg were used. Because of the relative freedom from rancidity of our diet X 113 and of the lower level of intake of tocopherol, the vitamin E requirements of the rabbits in this group were generally lower and varied within a narrower range, as would be expected if our assumption is correct. In the experiments with diet X 113 the requirement was found to range from 0.15 to 0.60 mg, and only few of the values so determined were outside the range of 0.20 to 0.40 mg per kg.

A few experiments were also made using the lipid extracted diet from which cod liver oil was also excluded. Instead of the cod liver oil additions, a few drops of 50% percomorph oil and a few drops of linseed oil were administered as separate supplements once a week. On such a diet the danger of vitamin E destruction is still further minimized.[2] Although these studies are only preliminary, even from the results so far obtained it seems very probable that the vitamin E requirement of these rabbits is actually only about one-half as great as was found to be the case in the other experiments.

These studies bring to light two important facts: (1) that the absorption from the gut may vary widely and that destruction in the gut or in the tissues, or in both, may be excessive resulting in some animals in a real vitamin E deficiency; (2) since synthetic dl-α-tocopherol suffices to prevent or to cure muscle dystrophy, components of the vitamin B complex may exert a favorable action[3] indirectly by protecting the vitamin E from destruction, possibly through an effect on the redox potential. Only the availability of synthetic α-tocopherol made a definite solution of the problem possible.

From our present experiments it seems probable that the rabbit requires 0.2 to 0.4 mg vitamin E per kg of body weight to cure and prevent the development of muscle dystrophy, and it is possible that, when more data become available by our improved dietetic technic, this value may be reduced even to about 0.1 mg.

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[2] Morris, S. G., *Science,* 1939, **90**, 424.

[3] Morgulis, S., Wilder, V. M., and Eppstein, S. H., *J. Nutrition,* 1938, **16**, 219.

11811 P

Development and Cure of "Ring-tailed" Condition in Rats on Vitamin B_6 Deficient Diets.

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From the Division of Animal Husbandry, University of California, Davis.

In the course of an experiment in which sheep and cow rumen contents were being assayed for vitamin B_6 by a modification of

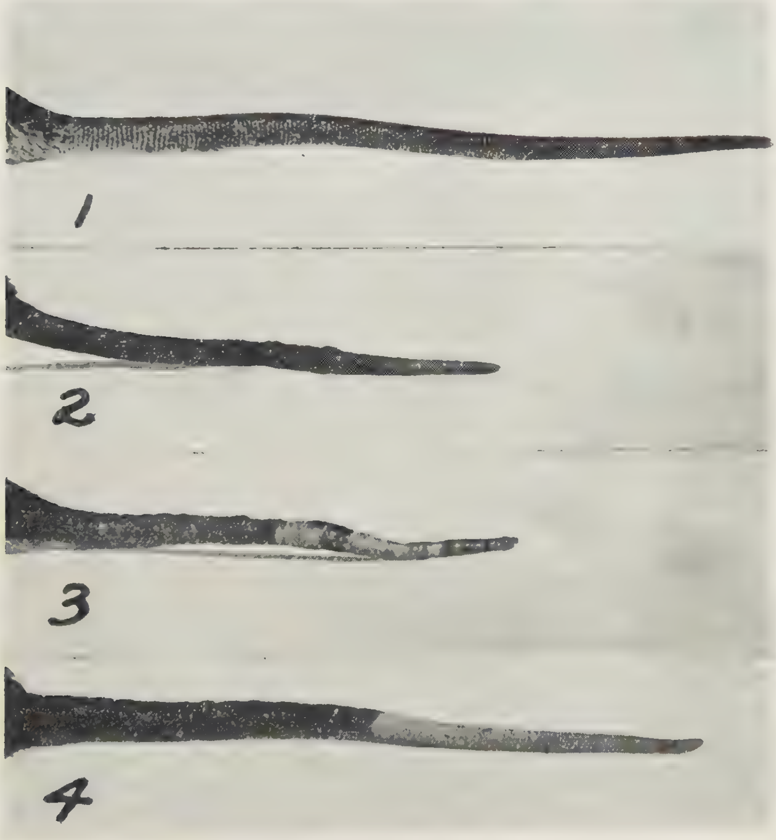

Plate I.

1. Tail of normal rat same age as 2 and 3.

2. Rat from basal group showing "ring-tail" lesions which began to appear after 42 days on the depletion diet. Photograph taken on 62nd day of depletion.

3. Rat from group supplemented with 10 μg of vitamin B_6 daily. There was no evidence of tail lesions on this rat when supplementation with vitamin B_6 was begun after 49 days on the depletion diet. Lesions appeared after 3 days of supplementation and became very severe by the tenth day, after which healing began. Photograph taken on the 14th day while the lesions were still distinct and the tail kinked.

4. Same rat as "3" after 27 days of supplementation with 10 μg of vitamin B_6, showing marked healing and growth of the tail.

the method of Dimick and Schreffler[1] we observed, in addition to the usual dermatitis of the face, ears and paws, ring-like (see plate I) lesions on the tail. This condition was first noted after the rats had been on the depletion diet for a period of 5 to 6 weeks and it developed in about 10 out of 100 animals used during the depletion period of approximately 7 weeks.

The basal diets used had the following composition:

	A	B
Glucose (cerelose)	60	60
Casein, washed with water at pH 4.5	27	27
Hydrogenated cottonseed oil (crisco)	8	4
Lard	—	4
Salt mixture[2]	5	5
Fish oil blend, 3000 U.S.P. units of vitamin A and 400 A.O.A.C. units of vitamin D per g	0.2	0.2

Thiamin chloride hydrochloride, 15 μg per rat daily.
Riboflavin, 30 μg per rat daily.

In the first assay, using diet A, factor II was supplied by feeding 0.5 cc daily of a liver filtrate concentrate prepared from Lilly Liver Extract Powder 343* by 5 treatments with fuller's earth after the method of Lepkovsky, Jukes and Krause.[3] The filtrate secured by this procedure was further treated with 8 volumes of 95% ethanol and the resulting precipitate was discarded. The final filtrate was concentrated under reduced pressure to the point where 1 cc represented 2 g of the original liver extract powder. In the second assay using diet B a filtrate was used which had been prepared from Armour's Liver Extract 70 A.S.† by similar treatment. This preparation was fed at a level of 0.2 cc daily since preliminary experiments showed that this amount supplied sufficient factor II to satisfy the growth requirement of the rat.

Basal diet B contained 4% hydrogenated cottonseed oil and 4% lard. This change was made to avoid the possibility of a deficiency of unsaturated fatty acids in the diet. Since no difference was noted in the response of the rats in the two assays it is believed that both diets were equally satisfactory.

The tail lesions which developed in rats on these diets were characterized by slight constrictions encircling the tail at intervals and bounded at first by slightly swollen scaly areas. In the continued absence of vitamin B_6 the condition remained practically unchanged over a period of 4 weeks but when the positive control groups were supplemented with 3 and 10 μg respectively of synthetic vitamin B_6 the condition was first aggravated and then cured. Within 2 to 5 days of supplementation the tail lesions appeared in an additional 15% of the animals and in the more severe cases the tails became marked by a series of constrictions and intervening wet swollen areas denuded of hair. The tail lost much of its flexibility and frequently became kinked in one or more places. The stage of pronounced swelling and stiffness lasted from 3 to 7 days and was followed by a period of healing and rapid growth of the tail. The dermatitis of the face, ears and paws disappeared at the 10 μg level, and improved at the 3 μg level several days before healing of the tail lesions took place.

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[1] Dimick, M. K., and Schreffler, C. B., *J. Nutrition*, 1939, **17**, 23.

[2] Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 180.

* Kindly furnished by Ely Lilly and Company, Indianapolis.

[3] Lepkovsky, S., Jukes, T. H., and Krause, M. E., *J. Biol. Chem.*, 1936, **115**, 557.

† Kindly furnished by the Armour Laboratories, Chicago, Illinois, through the courtesy of Dr. E. F. Pike.

The same series of symptoms was noted in the groups supplemented with dried sheep or cow rumen contents. The reaction to the pure vitamin and to the rumen contents was similar also in that while the most severe swelling occurred in those groups receiving the highest levels of the supplement, complete healing occurred most quickly in these same groups.

The experimental evidence does not demonstrate whether the effect of vitamin B_6 in this condition is direct or indirect. György[4] has shown that the addition of crystalline vitamin B_6 to a choline-low diet aggravates the development of cortical necrosis of the kidneys. It is possible that a similar relationship may be involved in the present experiments.

11812

Flavin-Dinucleotide in Eggs of the Sea Urchin, *Arbacia punctulata.*

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From the Lilly Research Laboratories, Indianapolis, Indiana, and the Marine Biological Laboratory, Woods Hole, Mass.

In the course of an investigation of the respiratory systems of various marine eggs, the eggs of *Arbacia punctulata* have been analyzed for flavin-dinucleotide by the following adaptation of the d-amino acid oxidase method of Warburg and Christian.[1]

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[4] György, Paul, and Goldblatt, H., *J. Exp. Med.*, 1940, **72**, 1.

[1] Warburg, O., and Christian, W., *Biochem. Z.*, 1938, **298**, 150.

Mature eggs were obtained and, when necessary, fertilized as previously described.[2] About 3 g of eggs, either unfertilized or taken at any desired time after fertilization and development at 20°C, were tightly packed by centrifuging at 2,000 times gravity for 10 minutes; they were then cytolyzed with 10 volumes of water, immediately put in a water bath at 80°C and held there with stirring for 10 minutes.

After cooling, the suspension was centrifuged and the cell debris and the supernatant fluid (made one-third saturated with ammonium sulfate) were each extracted 5 times, each time with 1 ml of liquid phenol at 40°C. With the initial addition of phenol to the supernatant fluid from the cytolysis, a precipitate of denatured protein appeared. This was centrifuged off and separately extracted 5 times with phenol. The combined phenol extracts were mixed with 2 volumes of ethyl ether; the flavin-dinucleotide was then extracted from the phenol-ether layer with 5 successive portions of water, the total water volume being made equal to that used in the cytolysis. Traces of phenol in this water layer were removed by repeated ether washes. The crude water solution was then concentrated and virtually freed of ether by several hours' exposure to suction in a vacuum desiccator over calcium chloride.

The flavin-dinucleotide activity was determined manometrically at 38°C, using a partially purified protein from lamb kidney[3] and d,l-alanine as substrate. The egg extracts gave zero oxygen uptake with d,l-alanine in absence of the protein. In each experiment a standard curve was run with 5 or 6 dilutions of partially purified yeast flavin-dinucleotide;[1] this material had been previously standardized against a sample of Warburg's mono barium salt of pure flavin-dinucleotide, kindly supplied by Dr. Eric G. Ball.

The results of 15 determinations by this method are given in Table I. Each determination was performed at least twice; the

TABLE I.
Flavin-dinucleotide Content, μg per g Wet Arbacia Eggs.

Exp. No.	Unfertilized	Fertilized 20 min	 10 hr	 24 hr
21 W 40	8.5	—	—	—
23 W 40	11.2	10.5	—	—
25 W 40	9.3	8.2	—	—
58 W 40	7.9	9.6	10.5	10.0
61 W 40	5.3	8.8	—	—
67 W 40	8.4	12.0	12.5	7.7

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[2] Clowes, G. H. A., and Krahl, M. E., *J. Gen. Physiol.*, 1936, **20**, 145.

[3] Negelein, E., and Brömel, H., *Biochem. Z.*, 1939, **300**, 225.

results of a group of experiments on any one sample fell within ±10% of the mean value. All determinations opposite a given experimental number were made on eggs from the same batch.

The lumiflavin reaction[1] was performed with the residues of 58W, 61W, and 67W, containing, in all, approximately 0.4 mg (5×10^{-7} moles) flavin-dinucleotide, as measured by manometric test. Approximately 4×10^{-7} moles lumiflavin were obtained.

Conclusions. These experiments appear to indicate that *Arbacia* eggs contain a flavin-dinucleotide similar to, or identical with, that found in other tissues.[1,4]

11813

Measures of Respiratory Activity With Resting Cells.*

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The "resting cell" technic has gained wide acceptance and has proved extremely useful in the study of bacterial respiration. Quastel and Whetham[1] who pioneered the application of the method, defined "resting bacteria" those which had been centrifuged, washed with saline, and finally well aerated. In the absence of a nitrogen source such cells do not proliferate, and hence observed responses are not believed to be complicated by growth phenomena. They state, "Such an emulsion is always employed under such conditions that growth does not occur. The reactions brought about under these conditions we consider to be reactions produced by the resting organism. . . . The term resting organism as we have defined it is useful for comparison with resting muscle and other tissues." It should be noted that there is nothing in this definition which precludes assimilation of a part of the substrate instead of its partial or complete oxidation. Working with *Esch. coli* and a few other species, Clifton[2] has demonstrated that assimilation usually occurs; Wilson[3] has provided evidence that resting cells of the rhizobia likewise assimilate part of the glucose furnished them.

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[4] Ochoa, S., and Rossiter, B. J., *Biochem. J.*, 1940, **33**, 2008.

* This work was aided by a grant from the Rockefeller Foundation.

[1] Quastel, J. H., and Whetham, M. D., *Biochem. J.*, 1924, **18**, 519.

[2] Clifton, C. E., *Enzymologia*, 1937, **4**, 246.

[3] Wilson, P. W., *J. Bact.*, 1938, **35**, 601.

Allison and Hoover[4] have questioned the commonly recognized application of the term "resting cell"; they propose that a "resting cell should be defined not as merely a nonproliferating cell but as a nonproliferating and nonassimilating cell." They suggest, for example, that cells of *Rhizobium spp.* grown on a carbohydrate-free medium (Wilson[3]) are high in nitrogen and will, upon the addition of a substrate, assimilate this substrate and utilize their excess nitrogen in a "growth process."

The chief criterion for a resting cell suspension has always been the linearity of uptake on the addition of substrate. Any true growth response should be accompanied by an increased *rate* of respiration. We have observed in hundreds of runs with numerous strains of rhizobia and with a wide variety of substrates (excepting the polyhydric alcohols and acetate which are oxidized by adaptive enzymes as will be described in a later publication) that respiration of the rhizobia grown on a carbohydrate-free medium has been consistently linear.

The chief basis for the claim of Allison and Hoover[4] that suspensions of washed cells from a carbohydrate-free medium "grow" when carbohydrate is added is that such cells have a higher Q_{O_2} than those grown on a medium containing sugar. Although the traditional Q_{O_2} based on dry weight is satisfactory for tissue in which the ratio of respiring tissue to storage material (such as fat or complex carbohydrate) remains reasonably constant, it becomes unreliable when this does not obtain. This fact was recognized by Rubner in his classical studies on the energetics of different species and led him to compare values based on nitrogen content rather than dry weight. Wilson[3] emphasized that the $Q_{O_2}(N)$[†] is more reliable than the Q_{O_2} for measuring the respiration of the rhizobia since the quantity of gum produced by many species varies with their growth conditions. This gum, which is extremely difficult to separate from the cell, increases the apparent dry weight and thus lowers the observed Q_{O_2}. Similar considerations have been advanced by Berenblum, Chain and Heatley[5] in support of their belief that nucleic acid phosphorus is the most reliable measure of the true respiring tissue in liver, kidney and other animal cells.

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[4] Allison, F. E., and Hoover, S. R., *Trans. Third Commission International Soc. of Soil Sci.*, 1939, Vol. A, 32.

[†] We have previously used q_{O_2} to designate O_2 uptake/hr/mg N, but the term $Q_{O_2}(N)$ used by Allison and his collaborators has more general application and is adopted here.

[5] Berenblum, I., Chain, E., and Heatley, N. B., *Biochem. J.*, 1939, **33**, 68.

TABLE I.
Influence of Composition of Media on Various Measures of Respiratory Activity of *Rh. trifolii* 209.

		Q_{O_2}	Q_{O_2}(N)	Q_{O_2}(P)	Q_{O_2}(C)	Q_{O_2}(cell)	Endogenous: % of glucose respiration	% N
Experiment I: Constant nitrogen, varying carbohydrate.								
1. 100 ml yeast extract, no sucrose	E*	12.5	100	1030	26.9	17.9	21	12.48
	G*	60.2	482	4900	130.0	86.4		
2. 100 '' '' '' plus .25% sucrose	E	17.0	176	1890	34.8	23.2	32	9.65
	G	52.9	548	5870	108.2	72.0		
3. 100 '' '' '' '' .5% ''	E	20.2	316	3600	42.2	44.5	62	6.4
	G	32.4	507	5810	67.7	71.5		
4. 100† '' '' '' '' 1% ''	E	19.8	331	3760	42.8	43.6	64	5.98
	G	30.9	517	5880	66.8	68.2		
Experiment II: Constant carbohydrate, varying nitrogen.								
5. 1% sucrose, 10 ml yeast extract	E	19.7	365	3330	51.3	81.7	64.5	5.4
	G	30.5	565	5760	79.5	126.5		
6. 1% '' 50 '' '' ''	E	22.0	363	3860	49.4	49.5	66	6.06
	G	33.1	550	5840	74.7	75.0		
7. 1%† '' 100 '' '' ''	E	18.9	288	3080	41.9	38.4	56	6.55
	G	33.6	512	5500	74.6	68.4		
Experiment III: Synthetic media.								
8. Allison's medium plus .25% sucrose	E	11.7	151	1990	28.5	31.1	30	6.46
	G	38.7	600	6600	94.5	103.1		
9. '' '' '' 1% ''	E	14.5	246	2700	34.0	37.6	36	5.91
	G	40.0	676	7420	93.5	103.5		

*E—endogenous. G—glucose.

†Note that these two media are the same; a comparison of values indicates how well results can be duplicated.

Q_{O_2}(N) mm^3 O_2 uptake/hr/mg N content.

Q_{O_2}(P) '' '' '' nucleic acid P content.

Q_{O_2}(C) '' '' '' total carbon content.

Q_{O_2}(cell) mm^3 O_2 uptake/hr/cell $\times 10^{10}$.

All measurements at 34°C, pH 6.5, M/45 phosphate buffer, M/150 glucose.

These points are illustrated by the data in Table I which summarizes the respiration rates of washed cells of *Rh. trifolii* (Wisconsin strain 209) grown for three days at 28°C on media containing variable amounts of yeast water and sucrose.‡ Comparisons have been made on the basis of dry weight, nitrogen content, nucleic acid phosphorus content, total carbon, and cell numbers. Dry weights were obtained by drying suspensions on tared watch glasses at 100°C. Nitrogen was determined by the semi-micro Kjeidahl analysis of Umbreit and Bond.[7] The method of Berenblum and Chain,[8] modified for use with bacterial cells, was employed for nucleic acid phosphorus. Total carbon was found by a semi-micro modification of the method of Friedemann and Kendall.[9] Cell counts were made with the Petroff-Hauser counter.

Respiration rates on a sodium succinate substrate (not included in Table I) were higher than when glucose was supplied to organisms grown on the carbohydrate-free medium and on the synthetic medium, but the rates on succinate were less than the glucose oxidation with all other suspensions.

As would be anticipated (Wilson[3]), the Q_{O_2} was dependent upon the gum production of the cells, and high endogenous respiration characterized the gummy cells from media containing carbohydrate. Organisms grown on the base medium without carbohydrate had a nitrogen content of 12.5% and a Q_{O_2} on glucose of 60.2, whereas organisms grown with 1% sucrose had a nitrogen content of 6% and a Q_{O_2} of 30.9; the spread in values is 95%. An identical range of 95% was obtained in the values based on carbon content of cells (66.8-130.0). In contrast the $Q_{O_2}(N)$ values ranged only from 482 to 548, a spread of 13%. The range in the $Q_{O_2}(P)$ of 4900 to 5880 represents a difference of 20%. The maximum variation in respiration per cell was from 68.2 to 86.4 or 26%. The endogenous respiration, as percentage of that in the presence of glucose, rose from 21%

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‡ The media were as follows: Media 1-7 contained the salt mixture of Medium 79 (Fred and Waksman[6]); 100 ml of double strength yeast water per liter plus variable quantities of sucrose were used in media 1-4. Variable amounts of yeast water plus 1% sucrose were used in media 5-7. Media 8 and 9 contained the salt mixture of Allison and Hoover[4] plus 0.4 g NH_4Cl and 0.1 μg biotin concentrate (0.4%) per liter. All media contained 2.5% agar and were adjusted to pH 7.0.

[6] Fred, E. B., and Waksman, S. A., *Laboratory Manual of General Microbiology*, New York, 1928.

[7] Umbreit, W. W., and Bond, V. S., *Ind. Eng. Chem. Anal. Ed.*, 1936, **8**, 276

[8] Berenblum, I., and Chain, E., *Biochem. J.*, 1938, **32**, 295.

[9] Friedemann, T. E., and Kendall, A. E., *J. Biol. Chem.*, 1929, **82**, 45.

for cells grown in absence of added carbohydrate (and hence little gum) to 64% for cells grown with 1% sucrose.

Examination of the data of Experiment II indicates that in the media in which the carbohydrate level was constant but with varying concentrations of yeast extract, the nitrogen content, and presumably gum content, differed little; hence all measures of respiratory activity were equally constant. All cells were high in gum, and the endogenous respiration was about 60% of that on glucose. In Experiment III the organisms were grown on synthetic media which were essentially those used by Allison and Hoover. The Q_{O_2} of these cells on glucose was approximately 40 at 34°C (29.6 at 28°C). In other experiments the following average values have been obtained with cells grown on Allison's medium containing varying levels of sucrose (glucose as substrate at 34°C): 0.25% sucrose—Q_{O_2}, 45, $Q_{O_2}(N)$, 553, % N, 8.0; 0.5% sucrose—Q_{O_2} 31.2, $Q_{O_2}(N)$, 510, % N, 6.1; 1.0% sucrose—Q_{O_2}, 35, $Q_{O_2}(N)$, 532, % N, 6.2. Allison and Hoover[4] report that their organism (*Rh. meliloti* 131) grown in such media had a Q_{O_2} on glucose at 28°C of 6 to 9. Aside from strain variation, we are unable to explain this discrepancy.

A noteworthy feature of the data in Table I is that cells grown on the modified Allison's synthetic media in general showed higher activity than those grown on the yeast extract media plus added carbohydrate. A synthetic medium is ordinarily to be preferred since it is more reproducible than are media using yeast extract, and though endogenous respiration of rhizobia grown on this particular one is greater than when grown in the absence of carbohydrate, it is not excessive. The chief objection to its use is the extreme difficulty encountered in separating cells from extraneous gum, when the carbohydrate content of the medium is greater than 0.25%.

In summary, it is recognized that assimilation occurs with suspensions of resting cells, but there is no definite evidence that the ordinary measurements made with these cells (O_2 uptake and CO_2 production) are concerned with any cell function other than respiration. The factor of assimilation should be considered in interpreting results, but as yet no convincing evidence has been brought forth that the occurrence of assimilation is a serious objection to the use of resting suspensions.

Conclusions. The $Q_{O_2}(N)$ has much to recommend it as the most suitable measure of respiration activity. With the rhizobia, for example, not only does it show the least variation of the measures employed, independent of the composition of the medium used for growth of the cells, but also it rests upon a Kjeldahl analysis which is probably the easiest and most accurate of the determinations made.

As long as respiration of washed cells remains linear with time, assimilation of a portion of the substrate should not seriously interfere with interpretation of the results.

11814

Production of Anti-Bacterial Agglutinins by Carp and Trout at 10° C.

WINSLOW WHITNEY SMITH.* (Introduced by Claude E. ZoBell.)

From the University of Southern California, Los Angeles.

In recent years costly losses to the salmon industries and trout hatcheries of middle Europe, Great Britain, and North America have been caused by a generalized bacteremia, the etiological agent of which is *Bacterium salmonicida*. These losses have raised the question as to the possibility of inducing immunity in brood stocks of these species by the use of bacterial vaccines.

There is no physiological characteristic of fish, amphibians, or reptiles known to the author that indicates that the reaction of these animals to the injection of a bacterial vaccine should differ from the response of the warm blooded vertebrates. Nevertheless, a search of the literature has failed to reveal a single attempt to immunize fish with vaccines. Indeed, there are very few studies reporting the production of any antibodies by cold-blooded animals upon the injection of foreign proteins, and most of these reports concern animals held at 20°C or above.

Five studies report the production of antibodies against red blood cells. Lazar[1] injected frogs with washed bovine erythrocytes and incited the development of agglutinin titers of 1:10 to 1:80 in some individuals. Many animals, however, were negative. Schwarzmann[2] also found that frogs developed hemoagglutinins on the injection of erythrocytes. Allen and McDaniel[3] held 2 groups of frogs at room temperature (22°C to 27°C) and 2 similar groups in the refrigerator (8°C to 10°C). One group at each temperature was injected with human erythrocytes. The only one of the 4 groups that developed hemolysins was the one injected with erythrocytes and held at room temperature. These workers concluded that only when the frogs were kept above 20°C did they develop antibodies. Wollman[4] confirmed these results in experiments which also indicated that temperature is a determining factor as to whether or not frogs will produce antibodies. Using sand turtles, Downs[5] has shown that 15 of 50 animals developed anaphylactic response to foreign erythrocytes and 9 of 19 developed precipitin titers of not over 1:100 within 3 weeks. The temperature of the turtles is not given.

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*Most of this study was subsidized by and carried out in the laboratories of the Wisconsin Conservation Department, Madison.

[1] Lazar, Erwin, *Wien. Klin. Wchnschr.*, 1904, 1057.

[2] Schwarzmann, L., *Z. f. Immunitatsf.*, 1927, **51**, 138.

[3] Allen, F. W., and McDaniel, C., *J. Immunol.*, 1937, **32**, 143.

Five other reports concern the production of specific anti-bacterial agglutinins in cold blooded vertebrates. Widal and Siccard[6] used the typhoid bacterium as an antigen and induced agglutinins in several frogs of 3 species, *(Rana esculenta, Rana usca,* and *Hyla viridis)* at temperatures from 12° to 24°C, 21° to 23°C, 27° to 33°C, and at 37°C. More rapid antibody production occurred at the higher temperatures than at the lower. Babes and Riegler[7] report the production of specific agglutinins in crucian *(Carassius vulgaris)* and in tench *(Tinca vulgaris)* within 24 hours by the injection with *Proteus piscidus versicolor*. The maximum titer observed was 1:50. Pliska[8] induced *Pseudomonas punctata* agglutinins in carp *(Cyprinus carpio)* by the injection of the bacterium intraperitoneally. The fish were held at 20°C and titers of 1:10,240 were attained in 6 weeks. Indeed, a higher titer was obtained in carp than rabbits. Pliska, also, cites the production of antibodies against certain vibrio strains by Bergman and by Aaser each using northern pike *(Esox lucius)*. He reports that Nybelin was able to induce antibody production at 17°C but not at 9°C in eels. Gee and Smith[9] subjected 3 painted turtles *(Chrysemys elegans)* and one snapping turtle *(Chelydra tricarinata)* to semi-weekly injections of *Bact. salmonicida* vaccine. These turtles were held at room temperature which fluctuated between 23°C and 28°C. No agglutinins were present in the blood when the experiment started. Each animal developed agglutinins within 6 months and the gradual increase of agglutinins was observed in 2 animals. The maximum titer observed was 1:2,560.

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[4] Wollman, E., *Rev. Immunol.* (Paris), 1938, **4**, 101.

[5] Downs, M., *J. Immunol.*, 1928, **15**, 77.

[6] Widal and Siccard, *Compt. rend. de la Soc. de Biol.*, 1897, **49**, 1047.

[7] Babes, V., and Riegler, P., *Centralbl. Bakt.* (etc.), 1903, Abt. II, Orig., **33**, 438.

[8] Pliska, F., *Centralbl. Bakt.* (etc.), 1938-39, Abt. I, Orig., **143**, 262.

[9] Gee, L. L., and Smith, W. W., *J. Bact.*, 1941, **41**, in press.

Twenty-seven adult carp *(Cyprinus carpio)* were placed in water having a temperature varying from 20°C to 23°C by Gee and Smith. The fish were given semi-weekly intraperitoneal injections of 1 ml of *Bact. salmonicida* vaccine (5 billion bacteria.). After 7 weeks these fish were removed to running water, the temperature of which was constant at ca. 10°C. Twenty-six of these carp developed agglutinins. Furthermore, 21 carp were tested more than once and, with one exception, each had an increase in titer as the injections continued. This increase also took place after the animals were removed to 10°C water. Scores of control-carp were shown to have no natural agglutinins for *Bact. salmonicida*. Incidentally, there was slight indication that these animals were immune to trout furunculosis, the bacteremia caused by *Bact. salmonicida*.

In all these experiments reporting antibody production, the temperature of the animals has been either not recorded or has been near room temperature. It will be noted that some of the workers report that no antibodies are formed at temperatures as low as 10°C, the temperature of good trout water.

To determine if antibodies can be formed by carp and trout at temperatures lower than those reported, 74 animals were subjected to strenuous courses of vaccination. The bacterial vaccine consisted of a physiological saline solution suspension of 24 to 72 hour carp-infusion peptone, agar culture of *Bact. salmonicida*, grown at 20°C and killed by heating at 60°C for 5 minutes. Each milliliter contained approximately 5 billion bacteria.

Five adult carp were placed in each of 3 tanks of 10°C running water. Five were injected bi-weekly, 5 weekly, and 5 semi-weekly, though all the fish received the same amount of vaccine, 2 ml, within a 2-week period. Most of the fish died within 12 weeks, because of confinement. Post mortem blood samples were titrated

TABLE I.

Titers of agglutinins of the blood of 74 fish held at 10°C and subjected to injections of suspensions of heat-killed *Bacterium salmonicida*. Each number in table represents the number of fish having the given titer at the time of death.

	Agglutinin titers							
Species	0	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
Carp	9*	2	1	2	—	—	1	—
Brown Trout	9*	8	2	5	1	2	1	—
Rainbow Trout	6*	4	9	9	1	—	1	1

*Nineteen of the 24 fish that died without showing agglutinin production died within 2 weeks of the start of the experiment.

for agglutinins. Of the 8 carp that survived until the tenth week, at death, 2 had titers of 1:20, 2 had titers of 1:40, one had a titer of 1:80, and one of 1:640 (see table). The remaining fish died during the first 2 weeks of the experiment and had no agglutinins. In earlier experiments it has been shown that carp in nature do not have these agglutinins.

The other fish, adult rainbow and brown trout, held in 10°C running water, were subjected to weekly injections of 1 ml of *Bact. salmonicida* vaccine containing approximately 5 billion heat-killed virulent bacteria. Because of the conditions under which they were held, few trout survived longer than 5 weeks, and most of them died during the second and third weeks. Post mortem blood samples taken after the first week showed agglutinins, the results being recorded in the table. It will be noted that a number of trout had no agglutinins; but most of these died early in the study. Three of 74 control animals were shown to have titers as high as 1:80. This does not necessarily indicate the presence of natural agglutinins. The presence of agglutinins for *Bact. salmonicida* in these trout, which had received no vaccine injections, is more readily explained by the fact that these animals have been exposed to trout furunculosis throughout most of their several years of life. It is very possible that the antibody has been developed in response to this natural exposure of the trout to the infection.

These experiments indicate that agglutinin production takes place at 10°C. It is believed that earlier investigators failed to detect this phenomenon because their animals were not given the antigen over a long enough period of time.

Experiments are now in progress with gold fish, which tolerate extremes of temperature, to determine the effect of temperature on the rate of agglutinin production.

11815

Comparison of Growth of *Trichophyton interdigitale* on Wool Fabric With and Without Additional Nutritive Media.

RUTH ELMQUIST ROGERS, DORIS J. HIRSCHMANN AND HARRY HUMFELD. (Introduced by Phoebe J. Crittenden.)

From the Bureaus of Home Economics and Plant Industry, U. S. Department of Agriculture.

In connection with the Department of Agriculture's investigations on the sterilization of wool,[1–3] studies have been made of the growth of *Trichophyton interdigitale* on wool. This fungus is one of the micro-organisms responsible for the ringworm infection "athlete's foot."

Gould and Carter[4] inoculated wool with *T. interdigitale* and at the end of 2 months observed no evidence of growth. On the other hand, Bonnar and Dreyer[5] who studied the thermal death periods for *T. interdigitale, Microsporon lanosum, Epidermophyton cruris,* and *Trichophyton rosaceum,* grew these organisms on undyed flannel and found that they had permeated the fabric and, in some cases, apparently digested it. Berberian[6] obtained growth of *T. interdigitale* on a woolen stocking after sterilization and inoculation with the spores of this organism.

Materials and Methods. The material used was an undyed, laundered, wool-blanket fabric which weighed 23.9 ounces per square yard and was 0.159 inch thick. For this study the fabric was cut into ½-inch squares, and each square was sterilized in a separate Petri dish at 15 pounds steam pressure for 30 minutes.

The isolate of *Trichophyton interdigitale* used was a transfer of No. 4808 of the American Type Culture Collection, 1939. An inoculum was prepared by growing this culture at room temperature on slants of Sabouraud's agar for 10 days. The growth was carefully removed from the slant, transferred to sterile distilled water, and shaken with glass beads. The suspension was then centrifuged and the sediment washed with sterile distilled water. This process was repeated 2 additional times, and the final suspension of the spores made in sterile distilled water.

Four media were used in this work: Sabouraud's agar, peptone agar, mineral salts agar, and water agar. The composition of the Sabouraud's agar was as follows: agar, 18 g; peptone, 20 g; dextrose, 40 g; and distilled water 1 liter. The formula for the peptone agar was: agar, 18 g; peptone, 20 g; and distilled water, 1 liter. The mineral salts agar contained: agar, 18 g; $NaNO_3$, 3 g; K_2HPO_4, 1 g; $MgSO_4$, 0.25 g; KCl, 0.25 g; and distilled water,

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[1] Humfeld, H., Elmquist, R. E., and Kettering, J. H., U. S. Dept. Agr. Tech. Bull. No. 588, 1937, 26 pp.

[2] Humfeld, H., Kettering, J. H., and Elmquist, R. E., U. S. Patent No. 2,204,360, 1940.

[3] Elmquist, R. E., Humfeld, H., and Kettering, J. H., U. S. Patent No. 2,207,630, 1940.

[4] Gould, G., and Carter, E. K., *Am. J. Hygiene,* 1931, **14**, 694.

[5] Bonar, L., and Dreyer, A. D., *Am. J. Public Health,* 1932, **22**, 909.

[6] Berberian, D. A., *Arch. Dermat. and Syphil.,* 1938, **38**, 367.

1 liter. The water agar consisted of: agar, 18 g; and distilled water, 1 liter.

All the media were adjusted to pH 5.8 by the colorimetric method using brom thymol blue and sterilized at 15 pounds steam pressure for 20 minutes. Each media was poured into sterile Petri dishes, 20 ml to each dish, and allowed to harden at room temperature. The plates were incubated 48 hours to test sterility.

Sabouraud's agar was considered a satisfactory nutrient media to support the growth of *T. interdigitale*. Peptone agar differed from Sabouraud's in that the carbohydrate carbon source, dextrose, was omitted. In the mineral salts agar, both dextrose and the nitrogen source, peptone, were omitted, but certain inorganic salts were added. The media which contained only agar and water was considered a non-nutritive base.

The sterile wool squares were transferred to the hardened agar plates by placing one piece of fabric in the center of each plate and pressing firmly against the media by means of sterile forceps. Ten squares were thus transferred to each of the 4 media, and 5 plates of each media were maintained as controls; that is, no fabric was added to the plate. Each plate containing the fabric was inoculated with a 0.1 ml suspension of the fungus by placing one-half of the suspension on the center of the wool squares and the remainder on the fabric edges. Each control plate also was inoculated in the center of the dish with the same amount of the organism. All plates were incubated for 14 days at room temperature.

Results and Discussion. All 5 controls of the peptone agar, as well as those of the Sabouraud's agar, furnished sufficient nutrient for the growth of *Trichophyton interdigitale,* but no growth occurred on any of the 5 replicates of the mineral salts media nor on those of the water agar media. Growth of the fungus was obtained on each of the 10 wool squares placed on Sabouraud's agar, on peptone agar, on mineral salts agar, and on water agar. These results are illustrated in Fig. 1, which shows the various controls and samples after incubation for 10 days at room temperature.

The results show that *T. interdigitale* was able to utilize wool fabric for its growth and multiplication in the absence of added dextrose or peptone, as evidence by its excellent growth on the fabric when either the mineral salts agar or the water agar were used. *T. interdigitale* apparently utilized peptone as a source for carbon and nitrogen, since peptone agar without wool supported a good growth.

The growth of *T. interdigitale* on wool was apparently stimu-

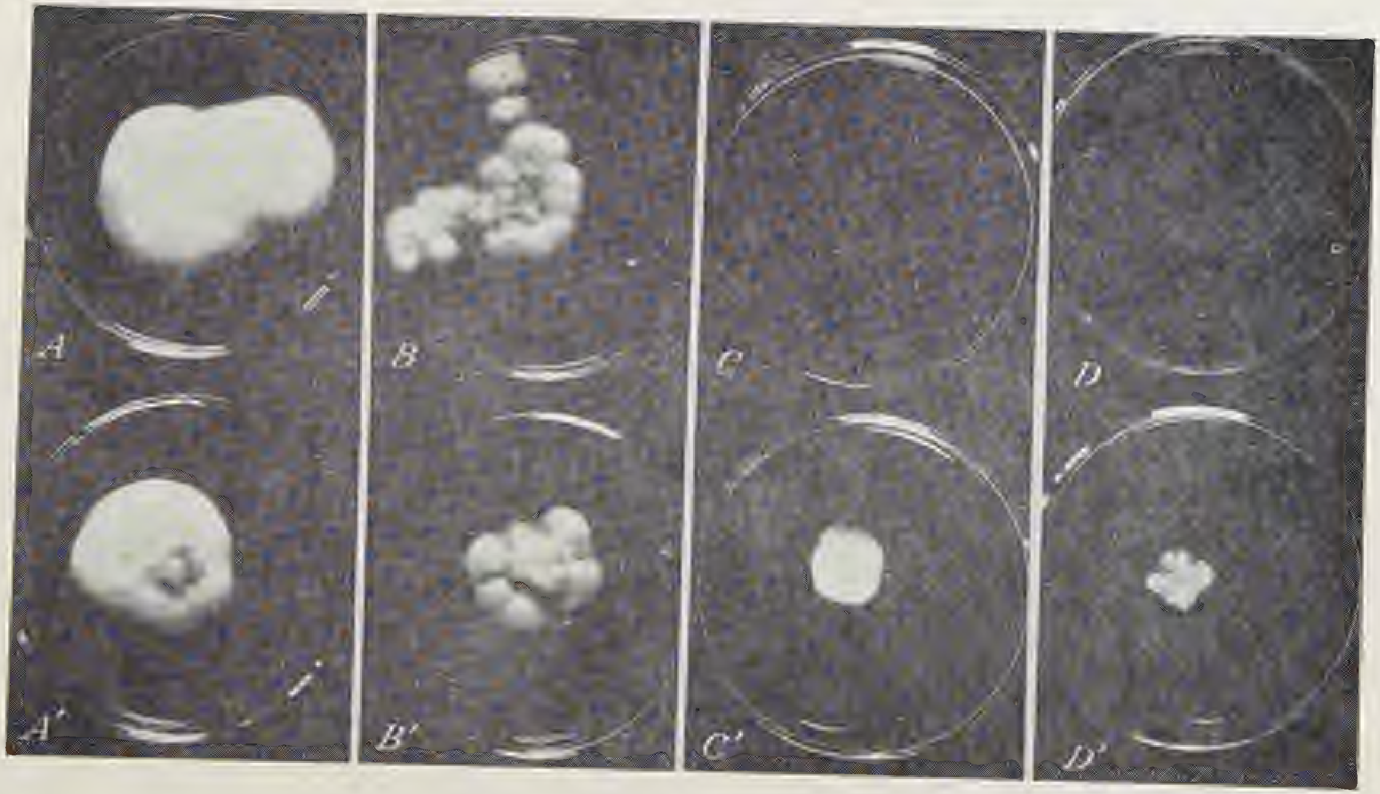

FIG. 1.

Growth of *Trichophyton interdigitale:* A, Sabouraud's agar without wool; A′, Sabouraud's agar with wool; B, Peptone agar without wool; B′, Peptone agar with wool; C, Mineral salts agar without wool; C′, Mineral salts agar with wool; D, Water agar without wool; D′, Water agar with wool.

lated by the addition of mineral salts. Examination of Fig. 1 shows increased growth of the fungus on the wool fabric where mineral salts agar was used in place of Sabouraud's agar, peptone agar, or water agar.

It was noted that the growth of *T. interdigitale* was slower on the wool when the mineral salts agar or water agar was supplied than when the Sabouraud's or peptone media were used. At the end of 4 days' incubation, growth was observed only on the two latter media.

Observation of Fig. 1 indicates that *T. interdigitale* utilized the media carbohydrate source of carbon in preference to the wool within a 10-day period. The growth on Sabouraud's agar without wool was profuse; whereas the growth on the wool placed on this agar was moderate. It is interesting to observe that this fungus showed the protein-sparing action of carbohydrates reported by Kendall[7] for bacteria.

When the dextrose is omitted, as in peptone agar, the wool was readily attacked and showed a good growth at the end of 10 days' incubation. At the end of 14 days, however, excellent growth also was observed on the fabric placed on the Sabouraud's media. This may indicate that after the media nutrient source is utilized, the wool offers a satisfactory substitute.

The findings reported here are of practical value, for wool hose are frequently worn by individuals suffering from "athlete's foot." These hose may be a constant source of infection since wool fabrics are not washed at sufficiently high temperatures, either in the home or in the laundry, to kill microorganisms. Legge[8] found that the most persistent cases of "athlete's foot" occurred when wool hose were worn. Also, because this fungus uses wool as a source of food, it causes deterioration of the fabric and hence decreases its serviceability. Possibly the mineral salt contained in perspiration and absorbed by wool hose aid in the growth of the fungus.

Summary and Conclusions. Trichophyton interdigitale was incubated on Sabouraud's, peptone, mineral salts, and water agar, both with and without the addition of wool. It was found that this fungus grew on the Sabouraud's and peptone media but not on the mineral salts or the water agar media. It grew on wool, however, when the wool was placed on all 4 media. Mineral salts appeared to stimulate the growth of the organism on the wool.

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[7] Kendall, A. I., *Physiol. Rev.*, 1923, **3**, 438.

[8] Legge, R. T., *Am. J. Public Health and the Nation's Health*, 1931, **21**, 648.

11816

Absorption and Excretion of Sulfadiazine.*

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Sulfadiazine,[†] the pyrimidine analogue of sulfapyridine, was synthesized by Roblin, Williams, Winnek, and English.[1] Its toxicity, absorption, and chemotherapeutic activity in experimental animals have been investigated by Feinstone, Williams, Wolff, Huntington, and Crossley,[2] who found that the drug caused less tissue damage and yielded higher blood concentrations than either sulfapyridine or sulfathiazole, and had a high therapeutic activity in pneumococcal, streptococcal, staphylococcal and Group B Friedlander's bacillus infections.

We have studied the effects of oral administration of single and of multiple doses of sulfadiazine in patients. Frequent estimations of the blood levels were made and the urinary excretion of the drug was determined by the method of Bratton and Marshall.[3]

Four patients were given a single 2 g dose of sulfadiazine. The average blood level for free and total drug is illustrated in Fig. 1. As can be seen, sulfadiazine is rapidly absorbed into the blood stream, and the average level attained exceeds that absorbed with the same dose of either sulfapyridine or sulfathiazole. The proportion of acetylated drug is small, and excretion by the kidney is relatively slow. At the end of 24 hours there is an appreciable amount of the drug in the blood, and at the end of 48 hours at least traces are still present. By the end of 12 hours, an average of .359 g of free and .202 g of conjugated sulfadiazine had been excreted in the urine;

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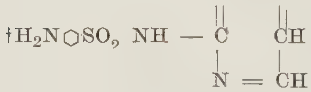
[*] This investigation was carried out under a grant from the Josiah Macy, Jr., Foundation. The sulfadiazine was supplied by Lederle Laboratories, Inc.

[†]



$$H_2N\bigcirc SO_2\ NH - \begin{array}{ccc} N & - & CH \\ \| & & \| \\ C & & CH \\ | & & | \\ N & = & CH \end{array}$$

[1] Roblin, R. O., Jr., Williams, J. H., Winnek, P. S., and English, J. P., *J. Am. Chem. Soc.*, 1940, **62**, 2002.

[2] Feinstone, W. H., Williams, R. D., Wolff, R. T., Huntington, E., and Crossley, M. L., in press.

[3] Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

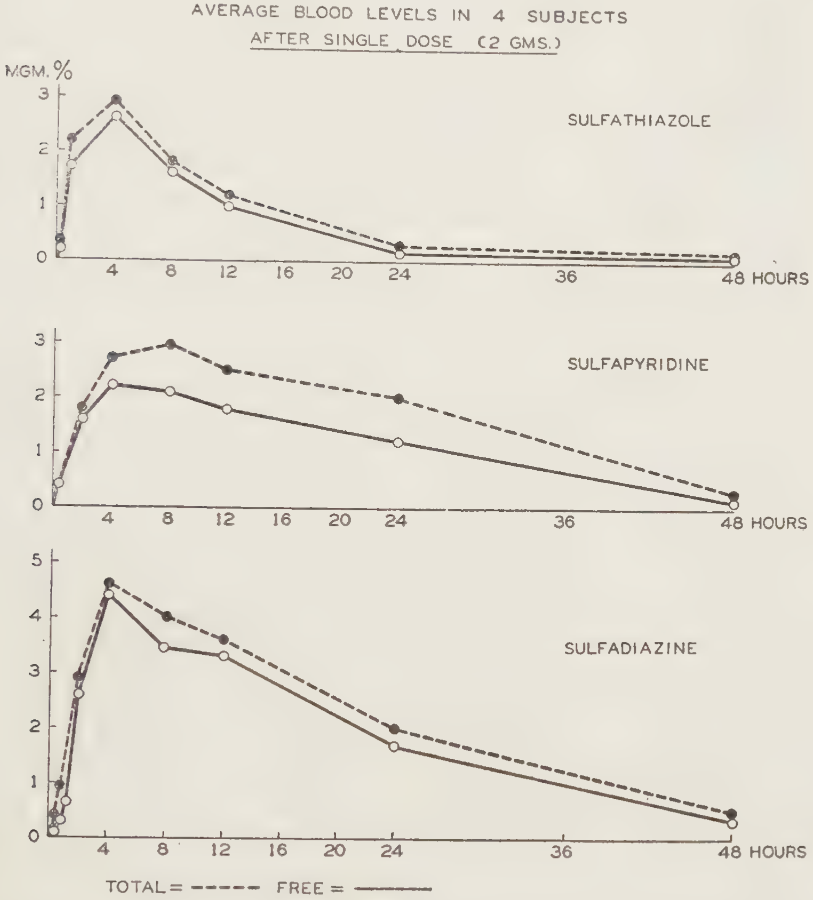


Fig. 1.

Average blood levels after single 2 g doses of sulfathiazole, sulfapyridine and sulfadiazine. Four different subjects were used to obtain each curve.

during the next 12 hours, an average of .215 g of free and .183 g of conjugated drug was excreted. In one case, .198 g of free and .240 g of conjugated sulfadiazine was excreted during the following 24 hours.

Eight patients were given 2 g of sulfadiazine as an initial dose followed by 1 g every 4 hours, in most cases until a total of 16 g had been given. The observations in these cases are tabulated in Table I. The average level of sulfadiazine in the blood is higher than that achieved with sulfapyridine or sulfathiazole in the same dosage. The proportion of acetylated drug is low. The blood level falls relatively slowly after the drug is discontinued, and from 40 to

TABLE I.

Blood Levels and Urinary Excretion Following Repeated Doses of Sulfadiazine (2 g, then 1 g Every 4 Hours).

Patient			Blood		Urine						Blood levels after stopping drug		
					Sulfadiazine								
							Excreted, g						
Init. Age Diag.	Wt in kg	Hr after admin.	Free	Total	Free	Total	Free	Total	Benzidine Test	Albumin	Hr after last dose	Free	Total
			Mg per 100 cc										
1. C.P.	59	1	T.	T.									
49		4	7.4	10.6									
Asthma		8	9.6	10.0									
		12	9.0	10.0	88.0	120.0	.845	1.152	Neg	0			
		24	10.2	12.8	200.0	296.0	1.000	1.480	,,	0			
		48	10.6	13.2	196.0	260.0	2.744	3.640	,,	0			
		72			117.6	161.6	1.564	2.149	,,	0			
											17	4.9	5.8
											29	2.5	4.0
2. N.F.	61	1	T.	T.									
22		4	1.4	2.5									
Bronchitis		8	3.4	3.5									
		12	5.6	5.7	13.2	19.0	.108	.156	,,	0			
		25	7.2	7.8	81.6	114.0	.971	1.356	,,	0			
		48	9.2	9.6	121.6	176.0	2.918	4.224	,,	0			
											16	4.6	5.4
											40	1.5	1.7
3. N.O.	80	1	0	T.									
34		4	3.0	3.7									
Radiculitis		8	4.0	4.1									
		12	5.0	5.1	43.8	45.9	1.092	1.147	,,	0			
		24	6.8	7.5	60.6	75.4	1.090	1.357	,,	0			
		48*	8.0	8.2	92.8	119.2	2.876	3.695	,,	0			
											18	4.3	4.5
											64	F.T.	F.T.
4. S.W.	60	1	0.4	2.7									
27		4	4.4	4.5									

Acute Tonsillitis		8	5.0	5.0									
		12	6.2	6.4	22.5	44.9	.6075	1.212	Neg	0			
		24	7.4	7.6	70.1	74.0	1.402	1.480	,,	0			
		48	8.8	9.0	44.8	119.2	.582	1.535	,,	0			
											18	4.0	4.1
											40	1.0	1.7
5. L.O.	85	2	2.7	3.5									
50		4	4.2	4.7									
G.C. Arthritis		8	5.6	5.7									
		12	5.8	7.6	117.6	126.8	.705	.761	,,	0			
		24	6.7	6.8	168.4	188.0	1.515	1.692	,,	0			
6. J.D.	49	1	1.4	1.6									
46		4	2.3	2.3									
Chronic Malaria		8	3.4	3.6									
		12	4.4	4.5	15.0	23.3	.182	.282	,,	0			
		24	4.8	5.7	27.0	43.0	.226	.361	,,	0			
											24	2.5	4.4
											52	1.0	1.3
7. P.T.	49	1	T.	T.									
40		4	3.5	4.0									
Rheum. heart dis.		8	2.2	3.7									
Inactive, no failure		12	6.4	8.1	75.4	93.0	.629	.776	,,	0			
		24	4.4	6.4	29.8	41.2	.278	.385	,,	0			
											24	3.0	3.4
8. B.H.	65	1	F.T.	T.									
35		7	4.2	4.3									
G.C. Arthritis		12	3.1	4.1	166.8	236.0	1.735	2.454	,,	0			
		24	5.9	8.6	92.0	106.4	.488	.564	,,	0			
		48	6.6	7.5	125.0	307.6	2.887	7.105	,,	0			
											16	3.8	4.4

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*Spinal Fluid at this time = Free, 4.1, Total 4.1 mg%.
F.T. = Faint Trace.
T. = Trace.

52 hours later there is still 1 mg % or over of free drug in the blood. During the first 12 hours, an average of .737 g of free and .254 g of conjugated sulfadiazine was excreted in the urine; during the next 12 hours .859 g of free and .213 g of conjugated, and during the next 24 hours, 2.40 g of free and 1.64 g of conjugated. In 2 cases, the urinary excretion was followed for 3 additional days. In one of these cases (S.W.), a total amount of 10.0 g of free drug and 2.63 g of conjugated drug was recovered out of the total of 16 g of sulfadiazine given during the first 2½ days; in the other case (B.H.), the total recoveries were 6.29 g of free drug and 6.49 of conjugated, the amount of sulfadiazine administered also having been 16 g.

Toxic Reactions. Each urine specimen obtained was tested for occult blood by the benzidine method, and for albumin. No blood or albumin was found in any specimen.

There was no nausea or vomiting in any patient, nor any evidence of kidney, liver, or peripheral nerve damage. The only toxic effect noted was the occurrence of a morbilliform rash in one patient (J.D.). For this reason, the drug was stopped at the end of 24 hours. The patient's face and eyelids were edematous during the next day but the reaction gradually subsided. He had previously had a similar rash after 4 g of sulfapyridine.

Summary. 1. Sulfadiazine was rapidly absorbed into the blood stream and comparatively high levels of total drug were reached. 2. The proportion of conjugated sulfadiazine in the blood was small. 3. The blood level falls rather slowly after the drug is discontinued. 4. Most of the drug is excreted by the kidney. 5. In 4 patients given 2 g of sulfadiazine and in 8 patients given a total of from 7 to 16 g the only toxic reaction noted was a morbilliform rash in one case in the latter group.

11817

The Fate of Testosterone in the Human.*

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When testosterone is administered to men with deficient testicular secretion, the principal androgen excreted in the urine is androsterone.[1,2] However, another substance, etiocholan-3(α)-ol-17-one, an isomer of androsterone, has also been isolated from the urine of a patient receiving injections of testosterone.[1] In a previous communication presence of this substance was indicated but not definitely established.[2] This communication is concerned with the isolation and identification of etiocholan-3(α)-ol-17-one from the urine of a patient receiving testosterone, thus confirming the original observation. The conversion of testosterone into androsterone in the complete absence of the testis forms the second part of this report.

Isolation of Etiocholan-3(α)-ol-17-one. Twenty-four liters of urine was collected from a patient who showed symptoms of deficient testicular secretion during a period when this individual received 30 mg of testosterone propionate† per day intramuscularly. The urine was treated by the method previously described.[2] That fraction of the urinary extract which contained the neutral ketonic substances was subjected to chromatographic adsorption using carbon tetrachloride as the solvent. Selective elution was carried out using mixtures of carbon tetrachloride and ethanol. The column was eluted with carbon tetrachloride containing 0.1%, 0.2%, and 0.3% ethanol. From the 0.2% ethanol fraction was obtained 150 mg of pure androsterone, melting point 179-181°C, after three recrystallizations from 95% ethanol.

From the 0.3% ethanol in carbon tetrachloride fraction, a reddish viscous oil was prepared which on sublimation at 3 micra pressure

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* This work was aided by grants from the Rockefeller Foundation, the Fluid Research Fund of Yale University School of Medicine, and the International Cancer Research Foundation. The last-named grant was made to Dr. James B. Hamilton of the Department of Anatomy.

[1] Callow, N. H., *Biochem. J.*, 1939, **33**, 559.

[2] Dorfman, R. I., Cook, J. W., and Hamilton, J. B., *J. Biol. Chem.*, 1939, **130**, 285.

† Testosterone propionate was furnished by Ciba Pharmaceutical Products, Inc., under the trade name Perandren.

and 120°C yielded a crystalline sublimate. This material was recrystallized from aqueous ethanol and yielded 40 mg of a compound which melted at 144-146°C. The benzoate melted at 161-163° while the acetate melted at 95-96°C. This compares favorably with previously published constants on etiocholan-3(α)-ol-17-one, M.P. 147-151°C, the benzoate M.P. 161.5-163.5°C, and the acetate M.P. 91-94.5°C.[1]

Conversion of Testosterone into Androsterone in the Male Castrate. The patient employed in this study was a male castrate aged 40 years, who had been castrated for the past 20 years. This individual received orally 60 mg of testosterone propionate daily for 5 days during which time all the urine was collected. The urine was treated in the usual manner to prepare a concentrate of the neutral ketonic compounds. This fraction containing the neutral ketonic compounds was sublimed at 1 micron pressure and 125°C. The crystalline sublimate weighed 15 mg and was slightly colored. After 3 recrystallizations from ethanol, the compound melted at 175-177°C. On 2 further recrystallizations, the melting point was increased to 180-181°C. The melting point of the benzoate was 172-175°C. Neither the melting point of the free compound nor its benzoate was depressed when mixed with authentic samples of androsterone and androsterone benzoate, respectively.

Discussion. The conversion of testosterone into androsterone and etiocholan-3(α)-ol-17-one is quite unique since it points to a rather unusual biological reduction; that is, in the reduction of the double bond in ring A at carbons No. 4 and 5, two isomers are formed. It seems that the enzyme systems participating in this reaction do not exhibit the usual specificity. Since we have been successful in isolating etioallocholanol-3(β)-17-one from the urine of guinea pigs after the administration of testosterone,[3] it appears likely that the lack of specificity extends also to the reduction of the ketone group at carbon 3.

The conversion of testosterone to androsterone in the castrate definitely rules out the testis as the only site of this reaction. It does not rule out, however, the possibility that the reaction may also proceed in this tissue.

Summary. The conversion of testosterone to etiocholan-3(α)-ol-17-one in addition to androsterone in the human has been confirmed. It has been demonstrated that the conversion of testosterone to androsterone may take place in the complete absence of the testis.

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[3] Dorfman, R. I., and Fish, W. R., *J. Biol. Chem.*, 1940, **135**, 349.

11818

Oxidation-Reduction Potentials of Inflammatory Lesions of the Skin.

Calvin C. Torrance.

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The low oxidation-reduction potential of the vitamin-C molecule has suggested that the function of this substance may be concerned with the regulation of the Eh in the animal body. Fluctuations in the ascorbic-acid concentration of inflammatory lesions of the skin induced by diphtheria toxin and by heat have been reported from this laboratory.[1] Such lesions, therefore, offered a means of studying the effect of alterations in the vitamin-C concentration of tissues upon their oxidation-reduction potential. Fildes[2] reported on the oxidation-reduction potential of the subcutaneous tissue fluid, as determined by indicator dyes. His findings indicated that while the Eh determined in the normal animal was above that at which tetanus spores could germinate, the injection of such substances as 5% calcium chloride or aleuronate caused the potential to fall to levels at which growth of these microörganisms was possible.

Purdy[3] and her colleagues investigated the electrical potentials of the skin of persons and reported a correlation between the oxidation-reduction potential of the skin and the basal metabolic rate in the range of from —10 to +13% of the normal metabolism. They reported, however, that these relationships did not hold when the circulation of the area under investigation was markedly accelerated or retarded.

Methods of Investigation. In the present studies, guinea pigs with white skins were clipped over the back and injected intradermally with diphtheria toxin diluted for the intracutaneous test of susceptibility to diphtheria in persons, or with the toxin inactivated by heat. The dose of active toxin used in these experiments, 0.1 cc, contained 0.02 of a minimum lethal dose. It induced erythematous lesions in twenty-four hours, which extended and became necrotic by the end of the third day. Similar lesions were induced by holding against the skin for 60 seconds the end of a Nessler tube, 2 cm in diameter, which contained water heated to 70°C.

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[1] Torrance, C. C., *J. Infect. Dis.*, 1940, **67**, 53.

[2] Fildes, P., *Brit. J. Exp. Path.*, 1929, **10**, 197.

[3] Purdy, C., Johnson, A. F., and Sheard, C., *Science*, 1931, **73**, 46.

The Eh values of the extracellular fluid of such lesions and of uninflamed skin were estimated by injecting a series of oxidation-reduction dyes[4] in both the oxidized and reduced states. The animal was destroyed one-half hour after the injection of the dyes and the injected areas were incised and examined. If the blue color which was first observed did not increase in depth on exposure to the air or on the addition of potassium ferricyanide solution, the dye was considered completely oxidized. If the original color observed when the test area was incised increased appreciably on exposure to the oxidizing solution, the dye was estimated as reduced by half, and as completely reduced if the area which contained no blue color when first observed became blue on swabbing with potassium ferricyanide. Gradations of color between were evaluated on this basis. Occasionally, if the reduced methylene blue solutions had been exposed to light prior to injection, the leuko dye was destroyed and the color did not return when the oxidizing solution was applied. In such instances the tests were repeated.

Results. The oxidation-reduction potentials of the extracellular fluid of the skin of 11 normal guinea pigs were determined. The tests were made at different times during a period of 2 years. Three or more dyes were used on each animal. In 5 of 7 guinea pigs thionine, one of the dyes injected, was almost completely reduced (Eh +0.002 volts). In the remaining 4, cresyl blue was reduced similarly (Eh —0.01 volts). Methylene blue was never reduced more than half (Eh +0.01 volts) in the three animals in which it was used. Indigo trisulfonate (Eh —0.02 volts) was very slightly reduced in 2 animals. While there appears to be some fluctuation in the Eh of the extracellular fluid of the skin of normal guinea pigs, the range in the animals tested never fell below —0.02 volts and the available data suggest that it is usually higher.

Heated diphtheria toxin was given intracutaneously to 5 guinea pigs. One was tested with the dyes within the first hour; the remainder at 24-hour intervals for 4 days. In 4 there was almost complete reduction of methylene blue (Eh —0.05 volts). In the fifth tested at the end of 2 days, not more than 50% reduction was observed (Eh +0.01 volts). However, the readings of the reactions on all animals with indigo tetrasulfonate were in agreement and consistent with an Eh at which methylene blue would be nearly completely reduced. The Eh of the extracellular fluid of the skin of guinea pigs injected with heated diphtheria toxin which induces no anatomical lesions would appear to be reduced below that of the uninjected animals, at least for the first 4 days after injection.

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[4] Torrance, C. C., *J. Bact.*, 1938, **35**, 339.

Diphtheria toxin in doses of 0.02 M.L.D. was injected similarly into 19 guinea pigs and the Eh of the extracellular fluid of the injected areas was determined at intervals of from one to 5 hours. In only 4 was an Eh recorded which was significantly different from that of the normal animals. The extracellular fluid of the skin of 2 of the 5 examined after 2 hours showed slight reduction of indigo disulfonate (Eh —0.065 volts). In one of 3 tested after 4 hours gallocyanine was completely reduced (Eh —0.04 volts), while in the only animal in which determinations were made after a 4½-hr interval indigo disulfonate was partially reduced (Eh <—0.065 volts).

The toxin was given similarly to 17 guinea pigs and the Eh of the extracellular fluid of the skin was determined at one-day intervals for 4 days. In 2 of the animals destroyed at the end of the first period, indigo monosulfonate (Eh —0.09 volts) and in one indigo disulfonate (Eh —0.065 volts) were slightly reduced, while in 3 others methylene blue was nearly completely reduced (Eh —0.05 volts). The Eh of the extracellular fluid of the skin of 2 remaining animals varied little from that of the uninjected guinea pigs. Two days after injection indigo monosulfonate was slightly reduced (Eh —0.09 volts) and indigo disulfonate nearly 50% reduced (Eh —0.125 volts) in one animal. In 2 others the potentials of the extracellular fluid did not appear to be significantly below that of methylene blue reduced (Eh —0.05 volts). On the third day in tests on one animal, indigo tetrasulfonate was completely reduced (Eh —0.10 volts), in another 50% reduced (Eh —0.08 volts). The extracellular fluid of the skin of a third at this time was found to have a potential between methylene blue reduced and indigo disulfonate oxidized. On the fourth day slightly less negative oxidation-reduction potentials were recorded (Eh —0.08, —0.06, —0.04 volts).

Reactions similar to the diphtheria toxin lesions were induced on the backs of 6 guinea pigs by heat and the oxidation-reduction potentials determined after one, 2, and 5 hours. In all 6 animals thionine was only 50% reduced (Eh +0.08 volts).

Discussion and Summary. While a certain fluctuation was found in the oxidation-reduction potential of the extracellular fluid of the skin of normal guinea pigs, the changes recorded following the injection of diluted diphtheria toxin or the application of sufficient heat to induce a mild inflammatory reaction may be significant.

The changes in the oxidation-reduction potential of the extracellular fluid of the inflammatory reactions of the skin of the guinea pigs tested did not appear to be associated with the loss of ascorbic acid, as reported in previous work.[1] Comparable losses were found in the ascorbic-acid concentration of the skin lesions induced by diphtheria toxin and by heat, but the change in the Eh of the extracellular fluid of the skin in the reaction induced by heat was in the direction of a more positive potential while that of the toxin reaction was toward a more negative potential than was observed in the normal animals.

11819

Nature of the Change in Resistance of Red Cells to Hemolysis.

Priscilla M. Porter. (Introduced by Eric Ponder.)

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It is known in hemolysis experiments that a suspension of red cells varies in its resistance to hemolysis, becoming more resistant the longer it stands. Whether this change is due to an inhibitory substance which is given off, or whether the cell membrane is altered, is not known.

We therefore did experiments to determine: (1) whether the phenomenon has any temperature dependence, and (2) whether an inhibitory substance can be detected in the supernatant saline of a red cell suspension which had been standing at 39° C for about 3 hours.

In the first experiment, a standard suspension (1 cc rabbit red cells in 20 cc of 1% saline) was hemolysed by varying dilutions of saponin (1-10,000 to 1-50,000) at 16-18°C and at 39°C immediately after making the suspension and again after the suspension had stood for one hour.

In the second experiment, a standard suspension was left in a water bath at 39°C for 3 hours. It was then centrifuged, and the supernatant saline was used as the 0.8 cc of saline in the hemolysis of a fresh cell suspension.

The first experiment showed that the resistance of the cell suspension increased by varying amounts (from 0.1% to 17%) during the hour experimental period. These values might be indirectly a measure of the quantity of an inhibitory substance given off by the cell suspension. This loss, however, does not depend on the temperature at which the suspension was hemolysed.

In the second experiment, there was essentially no difference in the hemolysis time between the experimental and the control tubes.

These results show that the resistance of a red cell suspension increases, but as no appreciable inhibitory substance can be recovered from the saline of a suspension which has been standing, this increased resistance is probably due to a change in the cell membrane. Such a change, possibly of the nature of disorientation in a highly oriented cell membrane, is not unlikely to occur.

11820

Effect on Body Growth of Small Doses of Testosterone Propionate Administered at Different Seasons.

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Since the observation that castration in the male albino rat was followed by growth inhibition,[1-6] studies have been carried out to determine whether growth could be stimulated by the injection of male sex hormones. The first attempts made with daily injection of 1 mg of testosterone propionate resulted, surprisingly enough, in growth depression.[7] This seeming paradox was considered to have resulted from the inhibition of the pituitary growth function induced by excessive hormonal dosage.[7-14]

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[1] Rubinstein, H. S., Abarbanel, A. R., and Kurland, A. A., *Endocrinol.*, 1939, **25**, 397.

[2] Donaldson, H. H., and Hatai, S., *J. Comp. Neurol.*, 1911, **21**, 155.

[3] Evans, H. H., and Simpson, M. E., *Anat. Record*, 1937, **35**, 36.

[4] Korenchevsky, V., *J. Path. and Bact.*, 1930, **33**, 607.

[5] Commins, W. D., *J. Exp. Zool.*, 1932, **63**, 573.

[6] Lawless, J. J., *Anat. Rec.*, 1936, **66**, 455.

[7] Rubinstein, H. S., Kurland, A. A., and Goodwin, M., *Endocrinol.*, 1939, **25**, 724.

[8] Hamilton, J. B., and Wolfe, J. M., *Endocrinol.*, 1938, **22**, 360.

[9] Steinach, E., and Holzknecht, G., *Arch. f. Entwicklungsmech. d. Organ*, 1917, **42**, 490.

[10] Bugbee, E. P., and Simmond, A. E., *Endocrinol.*, 1926, **10**, 360.

[11] Spencer, J., D'Amour, F. E., and Gustavson, R. G., *Endocrinol.*, 1932, **16**, 647.

Experiments were then begun with smaller quantities of this same substance and it soon appeared that for treatment beginning at 26 days of age, the optimal dosage for growth stimulation was 0.05 mg daily.[15]

During the past year, 4 different groups of animals were subjected to this treatment, first in order to substantiate our previous findings, then in order to determine the effect of climatic conditions on the growth response.

During this period of time a total of 102 animals were observed. Fifty-three of these were treated while the remaining 49 served as uninjected litter mate brother controls. Treated animals received daily subcutaneous injections of 0.05 mg testosterone propionate (Perandren) from 26-80 days. With the exception of injections, all animals were kept under similar conditions. Injections were begun in successive groups in October 1939, January 1940, April 1940 and July 1940 so that respective batches were observed during each of the 4 seasons.

Body weights were taken at weekly intervals and body lengths were observed at the end of the experiment. Data were considered statistically[16] and a "significance ratio" of 3.0 was arbitrarily chosen as an indication of "probable significance" in observed differences.

Results. The mean initial weight of all control animals was 40.6±0.7 g. This was not significantly different from the mean initial weight of the treated group (41.1±0.7 g). The final body weight of the treated animals (188.2±2.3 g) was 15.8±3.3 g heavier than the final body weight of the controls (172.4±2.4 g). This difference with its "significance ratio" of 4.8 is "probably significant." The mean final body length of the treated animals (196.±1.2 mm) was only 4.8±1.4 mm more than that of the controls (191.2±1.1 mm) but the "significance ratio" here was 3.4. The difference is, therefore, "probably significant."

Because of the significant difference between initial weights of the controls and treated animals of the January 1940 group (test animals, 39.3±1.6 g; controls, 33.2±0.9 g; difference,

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[12] Spencer, J., Gustavson, R. G., and D'Amour, F. E., PROC. SOC. EXP. BIOL. AND MED., 1931, **28**, 500.

[13] Spencer, J., D'Amour, F. E., and Gustavson, R. G., *Am. J. Anat.*, 1932, **50**, 129.

[14] McCullagh, D. R., and Walsh, E. L., *Endocrinol.*, 1935, **19**, 466.

[15] Rubinstein, H. S., and Solomon, M. L., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 442.

[16] Pearl, R., *Medical Biometry and Statistics*, Saunders, Philadelphia, 1930.

TABLE I.
Differences in Weight Gains Between Respective Control and Tested Groups Whose Treatments began at Different Times of the Year. S.R. indicates "significance ratio."

	Controls		Treated animals			
Groups	No. animals	Gain (g)	No. animals	Gain (g)	Diff. in gain	S.R.
Oct.	12	128.4 ± 3.7	12	148.6 ± 4.1	20.2 ± 5.3	3.8
Jan.	10	139.3 ± 3.5	6	163.9 ± 4.4	24.6 ± 5.7	4.3
Apr.	17	118.8 ± 6.3	17	126.8 ± 6.2	8.0 ± 8.0	1.0
July	14	130.6 ± 5.4	14	148.9 ± 3.9	18.3 ± 6.4	2.9
Totals	53		49		71.1 ± 12.9	
Mean difference in gain					17.8 ± 3.2	5.5

6.1±1.73 g; significance ratio, 4) comparisons were made between the weight gains of the all-treated and control groups.

From Table I it may be seen that all 4 treated groups gained more weight during the period of treatment than their respective controls. Taken as a whole the treated animals had gained 17.8±3.2 g more than their controls. This leads to a "significance ratio" of 5.5 which is statistically quite "significant." However, it will be noted that the gain observed in the April animals (significance ratio of 1) was not significant.

Discussion. From these results on experiments carried out throughout the year, it appears that testosterone propionate, in suitably low dosage definitely stimulates body weight and length. It is interesting to note, however, that although all animals were taken from the same strain, their initial weights varied tremendously with the season. For example, the initial weight of the October control group was 45.7±2.3 g (test=45.2±1.6 g); for the January controls it was 33.2±0.9 g (test=39.3±1.6); for the April controls it was 52.5±1.4 g (test=48.8±1.7 g), and for the July group it was 31.2±0.2 g (test=31.2±0.6 g). From this it appears that the growth up to 26 days was similar for the October and April groups and likewise similar for the January and July groups. On the other hand the October and April animals were markedly heavier initially than the January and July groups. It appears that the animals born in Autumn and Spring (October and April groups) had a naturally more rapid early growth period than the animals born in Winter and Summer (January and July groups).

This initial weight difference in control groups is perhaps the most important consideration prompting the comparison of weight gains rather than merely the final weights between control and test groups.

Those animals treated from April to July failed to show a definite growth response, remaining at the end in the same weight class as their controls. This seems to indicate that the result of treatment, like natural growth, is definitely influenced by climatic conditions. It appears from this study that the optimal season to begin growth studies of this nature in the albino rat is the Winter (January).

Summary and Conclusions. Testosterone propionate (Perandren) was subcutaneously injected through the 4 seasons into 4 respective groups of male albino rats in daily doses of 50 γ from 26 to 80 days. Final body weight in the treated animals was observed to be 188.2±2.3 g; of the controls 172.4±2.4 g. The difference of 15.8±3.3 g in favor of the treated animals (S.R.=4.8) was "probably significant." Final body length of the treated group (196±1.2 mm) was 4.8±1.4 mm longer than that of controls (191.2±1.1 mm). This was also "probably significant" (S.R.=3.4). Because of initial weight differences among the controls for the different seasons and because of a significant difference between the initial body weights of one treated group and their respective controls, comparisons were also made of "weight-gains" during the periods of observation. It was found that the treated animals gained 17.8±3.2 g more than their controls. This difference in weight-gain was considered to be statistically "probably significant" (S.R.=5.5). Although animals whose treatment began in April failed to show abnormally rapid growth those groups receiving injections during the Fall, Winter and Summer did become significantly larger than their respective controls.

It is therefore concluded that while animals generally respond to appropriately small doses of testosterone propionate with increased body weight and length, seasonal variation is a potent factor in the results obtained.

The authors gratefully acknowledge the coöperation of the Ciba Pharmaceutical Products Company, Inc., for partially defraying the expense of this study and for furnishing the testosterone propionate (Perandren) used.

11821

Menstruation-Like Bleeding with Constant Estrogen Dosage in an Ovariectomized Woman.*

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Corner[1] and Zuckerman[2] have reported recently that daily injection of a small amount of estrogen (*ca.* 30-60 units) into ovariectomized *Macacus rhesus* monkeys is associated with periodic menstruation-like bleeding which occurs during the period of treatment. Until then, the view was held generally that such bleeding depended upon estrogen-, or progesterone-withdrawal. There is no mention in the literature, however, of a result obtained in women comparable to that in the work of Corner and Zuckerman. The present note records such an observation.

This case is that of a woman, 38 years of age, who underwent bilateral ovariectomy and salpingectomy for cystic ovaries and meno-metrorrhagia. There was no infection, and the uterus was conserved *in toto*. The operation was performed on December 27, 1939, following a period of amenorrhea lasting for two years. This condition was terminated by persistent and excessive uterine bleeding following a course of injections of estrone and Antuitrin-S, in another clinic.

The patient came to the clinic at Greenpoint Hospital on February 21, 1940, for treatment of menopausal flushes. Five milligrams of estradiol-dipropionate (Di-Ovocylin, Ciba) were given twice weekly for 2 weeks, then once a week for 3 weeks. After this, 2 weeks elapsed between injections, and at the end of this time, on April 15, a period of uterine bleeding lasting 4 days took place. Pelvic examination showed the uterus to be normal in size, shape and consistency. At this point, a regular routine and constant dose of estrogen was instituted. It consisted of 2 mg of Di-Ovocylin given subcutaneously at intervals of 2 weeks, supplemented with one

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* Aided by grants from the Committee for Research in Problems of Sex, National Research Council, and the Josiah Macy, Jr., Foundation. The authors are indebted to Doctor Thurston Scott Welton for the privilege of conducting this work on his service in the Greenpoint Hospital.

[1] Corner, G. W., *Am. J. Physiol.*, 1935, **113**, 238.

[2] Zuckerman, S., *Proc. Royal Society of London*, Series B, 1937, **123**, 441.

tablet (0.5 mg) of estradiol (Ovocylin, Ciba) per day. This routine was maintained without variation until September 14, 1940. In the 5 months spanned by this period, menstruation-like bleeding occurred 3 more times, on May 2, May 29, and on August 4. Pelvic examination again showed the uterus to be normal. There was no further show of blood up to September 14, at which time the dose of estrogen was unavoidably altered.

Three periods of uterine bleeding were observed, therefore, the intervals between which (after the first induced period) were 18, 28, and 68 days, plus 31 days without bleeding until the observations were terminated. Thus this patient bled 3 times on constant estrogen-dosage, although the "cycles" were not regular. This is equally apt to be so in monkeys (see Corner's My. No. 102, in which the cycles were 26, 25, 15, and 44 days ([1], page 247); see also Zuckerman's My. No. OM78 ([2], page 453) in which the cycles were 48, 43, 35, 46, 31, 40, and 41 days). Even so, the present case would appear to offer sufficient similarity to the monkey data to render it certain that in woman, too, menstruation-like bleeding may occur under suitable circumstances on constant dosage of estrogen. There is, accordingly, no known difference between the monkey and woman in the methods by which menstruation may be experimentally induced.

11822

Inhibition of Respiration of Dysentery Bacilli by Sulfapyridine.*

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In a previous paper from this laboratory it has been shown that nicotinamide and some of its derivatives serve as essential growth factors for many dysentery bacilli.[1] More recently evidence has been presented which indicates that nicotinamide acts in some way other than, or in addition to, serving as a building block for one or both of the known phosphopyridine nucleotides.[2]

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The clinical use of nicotinic acid to control the nausea and vomiting resulting from the administration of sulfapyridine suggested to us the possible antagonism between sulfapyridine and nicotinamide.[3,4] After this work was under way, a paper by West and Coburn[5] appeared showing that sulfapyridine counteracted the growth-promoting activity of diphosphopyridine nucleotide (DPN) on *Staphylococcus aureus.*

Space does not permit a review of the literature concerned with the mechanism of action of sulfapyridine on microörganisms, but for the most part such work has concerned itself with the effect of sulfapyridine on the rate of multiplication of various microorganisms.[6] MacLeod[7] has shown that sulfapyridine inhibits the lactic, pyruvic, and glycerol dehydrogenases of sensitive strains of pneumococci. The ability to metabolize glucose was not affected.

A detailed description of the method of preparation of the cells used for the oxygen uptake measurements has been given elsewhere.[2] Briefly, the method consists of growing dysentery bacilli on a glucose containing synthetic medium deficient in nicotinamide. The cells are collected by centrifugation, washed twice with M/20 phosphate buffer (pH 7.4), and finally suspended in the phosphate buffer for use in oxygen uptake experiments. The direct method of Warburg is used for determining oxygen uptake.

It should be noted that the medium used in the respiration studies is free of nitrogen so that no chance of cell multiplication exists during the course of the experiments.

All tests were performed in duplicate and the essential experiments reported in this paper have been repeated several times. All comparisons of activity were made on the same suspension at the same time so that any variation in activity from day to day does not affect the results.

The dysentery culture used in these studies was of the Sonne type (strain 8).

Lederle sulfapyridine for standard solutions was used in all experiments.

It was found that the most consistent inhibitions were obtained

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TABLE I.
Effect of Sulfapyridine on Nicotinamide-Stimulated Respiration of Dysentery Bacilli.

	O_2 uptake for 2 hr minus blank (A), cmm O_2	% inhibition
A. 1.6 cc cell suspension	36.1	
B. 1.6 cc cell suspension + 0.8^2 nicotinamide in vessel	217.8	
C. 1.6 cc cell suspension + 0.8^2 nicotinamide in vessel incubated 1 hr, room temp. 800 γ sulfapyridine tipped in after incubation	188.7	13.3
D. 1.6 cc cell suspension. 0.8^2 nicotinamide tipped in after 1 hr incubation, room temp.	199.5	
E. 1.6 cc cell suspension + 800 γ sulfapyridine in vessel incubated 1 hr, room temp. 0.8^2 nicotinamide tipped in after incubation	26.7	86.6

0.3 cc of 2% glucose solution added to all vessels and all made up to a final volume of 2.7 cc.

if 800 γ of sulfapyridine were used in each vessel (30 mg %), and if the cells were allowed to stand with the sulfapyridine at room temperature for 1 hour before the nicotinamide was tipped in from the side-arm. The routine procedure adopted was to put the vessels and manometers in the bath exactly 50 minutes after the cells and sulfapyridine were mixed. After a 10-minute equilibration the stopcocks on the manometers were closed and the material from the side-arm was tipped into the main part of the vessel.

Table I is illustrative of a typical experiment. The oxygen uptake figures given are averages of duplicate determinations. Combination A is the blank, that is, the respiration of the deficient cells without the addition of nicotinamide. Numerous experiments have shown that this residual respiration is not inhibited by the addition of sulfapyridine. Combination B illustrates the effect of the addition of nicotinamide to deficient cells. It will be noted that the respiration is markedly increased. In this case the nicotinamide is added to the vessel together with the cells prior to incubation. The addition of sulfapyridine after incubation with nicotinamide (combination C) results in a small inhibition, which is just outside the range of experimental error. This inhibition varied from about 8 to 15% in different experiments.

Combination D again illustrates the stimulation due to nicotinamide, but in contrast to B the nicotinamide is tipped in after the incubation period. It will be noted that the oxygen uptake of D is somewhat lower than that of B. This decrease in activity on incubation has always been observed and is probably due to the gradual inactivation of the free enzyme during the period of incubation. The previous addition of nicotinamide seems to protect the enzyme. Combination E illustrates the effect of sulfapyridine when it is added before the nicotinamide. It will be noted that the inhibition is very marked in this case. Under these conditions the inhibition has always been striking although the percentage inhibition has varied from 80 to 95% in different experiments. This consistency was not obtained with smaller concentrations of sulfapyridine and without the period of incubation.

Table I strikingly illustrates the apparent antagonism between the action of nicotinamide and sulfapyridine. Experiments are now in progress to determine quantitatively the nature of this antagonism.

The results shown in Table I raise the question of the effect of simultaneous addition of sulfapyridine and nicotinamide. The results of numerous experiments on this point show a wide variation, but on the whole there seems to be a definite inhibition with a value between that obtained in C and E in Table I.

When sodium lactate is used as substrate in the Warburg vessels results qualitatively similar to those obtained with glucose are obtained. The percentage inhibition is somewhat smaller with lactate as substrate than with glucose.

Respiration stimulated by DPN is inhibited by sulfapyridine in a manner which is at least qualitatively similar to the inhibition illustrated in Table I.

These results strongly indicate that the action of sulfapyridine on microörganisms may be related to the role of nicotinamide in their metabolism.

Summary. It has been found that sulfapyridine in concentrations of 30 mg % inhibits the stimulation of respiration due to nicotinamide. This inhibition is apparently competitive since the sulfapyridine must be added prior to the addition of the nicotinamide to obtain this effect. While percentage inhibition has varied in different experiments, sufficient experimental data have been obtained so as to leave no doubt as to the validity of the inhibition.

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Total Carbohydrate and Glycogen Content of the Developing Honeybee.*†

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It has been reported that the concentration of total reducing substances, calculated as glucose, increases during the larval stage of the queen and worker honeybee (*Apis mellifera* L.) and decreases during pupation.[1] A further study presented here deals with changes in the total carbohydrate, glycogen, and free reducing sugar that accompany the development of the worker honeybee.

A review of some of the earlier work shows that investigators have been able to establish certain significant facts concerning the carbohydrate metabolism of the honeybee. Straus[2] reported that toward the end of the larval period of the worker honeybee more than 30% of the dry weight was glycogen, whereas Parhon[3] found the adult to contain but 0.14-0.21%. Straus[2] was able to detect little, if any, reducing sugar in the tissues of the developing worker bee; however, Sturtevant[4] found reducing sugar in 60% alcohol extracts of bee larvae. Moreover, both glucose and glycogen were found in the hemolymph of the prepupal honeybee by Ronzoni and Bishop.[5]

Experimental. Total carbohydrate, glycogen, and free reducing sugar were determined at 24-hour intervals during the life cycle of the honeybee. The methods of rearing insects and preparation of tissue for analysis have been described.[6] Worker honeybees complete their development in 18 days after hatching; feeding occurs during the first 5 days of larval life; pupation takes place on the 8th day of larval life; and the imago emerges 10 days later.

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† A contribution from the Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture, in coöperation with Louisiana State University.

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For the first 2 days after hatching the larvae are fed royal jelly, a secretion of the pharyngeal glands of the workers. During the third day the diet of the worker larvae is altered to include pollen and honey.

Total Carbohydrate. Total carbohydrate includes free sugar of the tissues, unassimilated carbohydrates in the alimentary canal, glucose produced by hydrolysis of the glycogen, and other carbohydrates convertible by acid hydrolysis into reducing sugar. In determining the total carbohydrate approximately 1 g of macerated tissue was placed in a 50-cc centrifuge tube, and 10 cc of 1.2 N sulfuric acid was added. The tube was closed with a rubber stopper that was provided with a 2-foot glass tube to serve as a reflux condenser. The tubes and their contents were heated for 3 hours in a boiling-water bath. When the digests were cool, they were neutralized with a saturated aqueous solution of barium hydroxide (phenolphthalein being used as an indicator), diluted to 50 or 100 cc and filtered. The solution was analyzed for its reducing power before and after yeast fermentation by the dinitrosalicylic acid method of Sumner[7] or the iodometric titration of reduced copper.[8]

Glycogen. The glycogen determinations were made by the Good, Kramer, and Somogyi modification of the Pflüger method.[9] The glycogen was hydrolyzed by heating for 2.5 to 3 hours with 5 cc of 1 N sulfuric acid. The acid hydrolyzates were neutralized with a saturated aqueous solution of barium hydroxide, phenolphthalein being used as an indicator. The solution was analyzed for its reducing power before and after yeast fermentation by the method of Sumner[7] or Somogyi.[8]

Free Reducing Sugar. The procedure of Steiner, Urban, and West[10] was used to prepare tissue filtrates for the determination of free reducing sugar in the body tissues of the developing honeybee. The reducing power of the filtrates before and after yeast fermentation was determined by Somogyi's method.[8]

Yeast Fermentation. Benedict's method[11] was used for preparing yeast for the fermentation of filtrates. About half a yeast cake was thoroughly mixed with 40 cc of distilled water in a 50-cc centrifuge tube, and the mixture was centrifuged for 3 minutes. After 4 such washings, the yeast was suspended in 50 cc of water, and 10 cc aliquots were transferred to 15 cc centrifuge tubes. After being centrifuged, the tubes were inverted on sheets of filter paper to drain for a few seconds, and the adhering film of moisture on the inside of the tube was removed by wiping with filter paper. The tissue filtrate was added and mixed with the yeast. Fermentation was allowed to proceed for approximately 30 minutes, after which the mixture was centrifuged and the reducing power of the filtrate determined.

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[8] Somogyi, M., *J. Biol. Chem.*, 1926, **70**, 599.

[9] Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 485.

[10] Steiner, A., Urban, F., and West, E. S., *J. Biol. Chem.*, 1932, **98**, 289.

[11] Benedict, S. R., *J. Biol. Chem.*, 1928, **76**, 457.

Results. The results from the analyses for total carbohydrate, glycogen, and free reducing sugar are presented in Table I. Each value represents the average of 6 determinations.

Discussion. Table I shows that during the larval stage the total carbohydrate and glycogen increase. The maximum storage of both of these materials occurs between the fifth and sixth days, which is approximately the end of the larval feeding period and the time the cell is sealed. The total carbohydrate at this time is 100 mg and the glycogen 68 mg per gram of tissue; however, 12 days later the total carbohydrate has decreased to 9 mg and the glycogen to 0.9 mg per gram. If it may be assumed that the daily rate of utilization is constant, the total carbohydrate decreases approximately at the rate of 8 mg and the glycogen at the rate of 6 mg per gram per day.

It is of interest that the synthesis and utilization of total carbohydrate and glycogen parallel each other throughout the life cycle. However, the glucose due to hydrolysis of glycogen varies at different stages of development; for example, at the 3-4-day stage it comprises 42% of the total carbohydrate, at the 5-6-day stage

TABLE I.
Total Carbohydrate, Glycogen, and Free Reducing Sugar of the Developing Worker Honeybee.

Age days	Stage	Expressed as mg of glucose per g of tissue		
		Total carbohydrate	Glycogen	Free reducing sugar
3-4	Larval	24	10	5
4-5	”	77	50	3
5-6	”	100	68	1
6-7	”	89	66	0.3
7-8	”	82	65	0.5
8-9	Pupal	75	57	0.8
9-10	”	66	51	0.9
10-11	”	59	47	0.9
11-12	”	55	42	1.0
12-13	”	50	35	1.0
13-14	”	38	25	1.3
14-15	”	31	19	1.5
15-16	”	26	12	1.8
16-17	”	19	10	2.0
17-18	Imagal	9	0.9	1.5

68%, and at the 17-18-day stage 10%. At no time does the free reducing sugar make up the difference existing between the total carbohydrate and glycogen. The greatest difference is 32 mg per gram of tissue at the 5-6-day stage, but at this time the free reducing sugar is only 1.0 mg per gram. The free reducing sugar decreases to 0.3 mg per gram at the 6-7-day stage, rises to 2.0 mg per gram by the 16-17-day stage, and decreases before emergence. It is clearly shown, from the results presented here, that some form of carbohydrate other than glycogen and free reducing sugar must be present. This carbohydrate is convertible, by acid hydrolysis, to reducing sugar. It is either destroyed by the alkali used in the glycogen procedure or not precipitated by the concentration of alcohol employed in the glycogen method.

The rapid synthesis of carbohydrate reserves may be accounted for by the presence of readily assimilable carbohydrate, chiefly glucose and levulose, in the diet of the developing worker honeybee. According to Snodgrass,[12] one of the important functions of the fat body of some insects is glycogen storage. In this respect the function is similar to that of the vertebrate liver. Glycogen prepared from prepupal worker honeybees gave a wine-red color with Lugol's iodine solution. Melampy and Willis[6] found the respiratory quotient of the worker honeybee to be greater than unity during the larval period, thus indicating a synthesis of lipid from carbohydrate at that time. The average respiratory quotient during the pupal period of the worker honeybee was 0.96.

The results presented here are in agreement with the findings of other investigators dealing with the carbohydrate metabolism of the developing honeybee. There is close agreement with the data of Straus[2] as regards glycogen, but, in contrast to his findings and in agreement with those of Sturtevant,[4] this investigation indicates the presence of free reducing sugar during development. The carbohydrate reserves of the developing honeybee are probably utilized in the production of energy and in the synthesis of chitin.

Summary. The changes in the concentration of the total carbohydrate, glycogen, and free reducing sugar have been investigated during the larval and pupal development of the worker honeybee. The following results have been obtained:

1. During larval development there is a marked increase in the total carbohydrate and glycogen.
2. During the pupal period both the total carbohydrate and

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[12] Snodgrass, R. E., *Anatomy and Physiology of the Honeybee*, McGraw-Hill, 1925.

glycogen decrease. At the time of emergence a very small amount of carbohydrate reserve remains.

3. In addition to glycogen, there is in the tissues of the immature honeybee a reserve carbohydrate which is convertible, by acid hydrolysis, to reducing sugar.

4. Free reducing sugar is found in the tissues of larval and pupal honeybees.